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(54) Title: DETECTION AND TREATMENT OF ATHEROSCLEROSIS BASED ON PLASMA SPHINGOMYELIN CONCENTRATION

(57) Abstract: Disclosed are new enzymatic methods of plasma and tissue sphingomyelin concentration measurement. Also disclosed is that human plasma sphingomyelin levels are strongly positively correlated with atherosclerosis and coronary heart disease. Thus, the use of a quick and effective plasma sphingomyelin measurement such as the subject invention, is valuable for screening assays in vitro, in cell culture or in animals to develop drugs or other treatments aimed to lower plasma sphingomyelin levels. The findings indicate that therapies aimed at reducing plasma or tissue SM levels are likely to have therapeutic benefit. These would include inhibition of sphingomyelin synthesis in the liver or arterial wall, as well as methods to enhance clearance of sphingomyelin from plasma. Thus, compounds which inhibit sphingomyelin biosynthesis or induce sphingomyelin clearance are also disclosed.

DETECTION AND TREATMENT OF ATHEROSCLEROSIS BASED ON PLASMA SPHINGOMYELIN CONCENTRATION

The invention described herein was made in the course of work under Grant Number HL56984 from the National Institutes of Health, U.S. Department of Health and Human Services. Accordingly, the U.S. Government has certain rights in the invention.

Throughout this application, various references are identified by authors and full citations. Disclosure of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains.

Background of the Invention

FIELD OF THE INVENTION

Only a fraction of the clinical complications of atherosclerosis are explained by known risk factors. Animal studies have shown that plasma sphingomyelin (SM) levels are closely related to the development of atherosclerosis. SM carried into the arterial wall on atherogenic lipoproteins may be locally hydrolyzed by sphingomyelinase, promoting lipoprotein aggregation and macrophage foam cell formation.

DESCRIPTION OF RELATED ART

The association of lipid abnormalities and atherosclerosis is well established. Case-control and prospective epidemiological studies have shown a direct correlation between coronary heart disease (CHD) and serum levels of total cholesterol and low density lipoprotein cholesterol (LDL-C), and an

inverse relationship between the CHD and high density lipoprotein cholesterol (HDL-C) levels (1-4). However, compared to plasma cholesterol, much less attention has been given to the relationship between phospholipids and coronary heart disease. In one report, high concentration of saturated phosphatidylcholine (PC) in plasma was a significant risk factor for atherosclerosis, independent of triglyceride and cholesterol levels (5). In another small study, HDL phospholipids correlated better with the severity of coronary heart disease than HDL-C (6).

Atherogenesis is initiated by the interaction of cholesterol-rich lipoproteins, such as LDL, with the arterial wall (7,8). The uptake of lipoprotein cholesterol by macrophages, leading to foam cell formation, is a central event in the initiation and progression of atherosclerosis (9). However, native LDL is incapable of generating foam cells from macrophages. Thus, it is thought that LDL is modified in the arterial wall, by processes such as oxidation, leading to macrophages chemotaxis and uptake of modified LDL by macrophage foam cells (10,11). Retention of lipoproteins on the subendothelial matrix, followed by aggregation, has also emerged as a central pathogenic process in macrophage foam cell formation and atherogenesis (12). Lipoprotein aggregation in the vessel wall may result from enzymatic modification of LDL, induced by locally produced sphingomyelinase (Smase) (12,13).

It has long been known that sphingomyelin ("SM") accumulates in human and animal atheroma, and that the major source is plasma lipoproteins (14-22). Plasma SM levels are increased in human familial hyperlipidemia, especially in familial hypercholesterolemia (23,24), and also in animal

models of atherosclerosis (25-27). The concentration of SM relative to total phospholipids (principally PC and SM), i.e. SM/(SM+PC), is an important determinant of the susceptibility of lipoprotein SM to Smase (27,28). These findings suggest that plasma SM levels and the relative SM concentration might be risk factors for atherosclerosis. However, plasma SM levels have never been systematically assessed as a risk factor for atherosclerosis in human.

This is partly due to the difficulties inherent in the classical method for SM measurement, which involves lipid extraction and thin layer chromatography (29,30,58). To overcome this difficulty we disclose a novel enzymatic method for plasma SM determination, and this method was used to measure SM in plasma samples from an angiographic coronary artery disease case-control study.

There are two major and three minor phospholipids in human plasma: phosphatidylcholine, sphingomyelin, phosphatidylinositol, phosphatidylethanolamine and lysophosphatidylcholine. Up to 18% of total phospholipids in plasma is sphingomyelin (31). Sphingomyelin is found in plasma membranes and related organelles (such as endocytic vesicles and lysosomes) of all tissues, as well as in lipoproteins (32). Sphingomyelin (SM), together with free cholesterol and phosphatidylcholine (PC), forms a phospholipid monolayer at the surface of plasma lipoproteins, and the ratio of SM/PC varies widely among various lipoproteins (33,34). Plasma lipoprotein SM content may be important in atherogenesis because the ratio of SM to PC is increased 5-fold in very-low-density lipoprotein (VLDL) from hypercholesterolemic rabbits (35). Apolipoprotein E-deficient mice (apoE KO), an atherosclerosis-prone mouse model, showed an increase

of SM concentration in LDL and VLDL which predisposed these particles to be aggregated after mammalian sphingomyelinase treatment (36). A related arterial wall sphingomyelinase has been prepared to enhance atheroma foam cell formation by inducing aggregation and cellular uptake of SM-rich lipoproteins. These results suggest that sphingomyelin may play a critical role in the development of atherosclerosis. However, classical methods for SM measurement are limited in their accuracy, quickness, and ability to perform on a large scale, such that epidemiological evidence for the relationship between human plasma sphingomyelin and atherosclerosis is still lacking.

Summary of the Invention

Disclosed are new enzymatic methods of plasma and tissue sphingomyelin concentration measurement. Also disclosed is that human plasma sphingomyelin levels are strongly positively correlated with atherosclerosis and coronary heart disease. Thus, the use of a quick and effective plasma sphingomyelin measurement such as the subject invention, is valuable for predicting coronary artery disease and atherosclerosis susceptibility in humans, and for screening assays in vitro, in cell culture or in animals to develop drugs or other treatments aimed to lower plasma sphingomyelin levels. The findings indicate that therapies aimed at reducing plasma or tissue SM levels are likely to have therapeutic benefit. These would include inhibition of sphingomyelin synthesis in the liver or arterial wall, as well as methods to enhance clearance of sphingomyelin from plasma. Thus, this invention also provides compounds which inhibit sphingomyelin biosynthesis or induce sphingomyelin clearance.

Brief Description of the Figures

Figure 1. The plasma sphingomyelin assay begins with four sequential enzymatic reactions and leads to the production of a pigment which is then measured, and related to a standard curve, giving the measurement of plasma sphingomyelin levels. The four sequential enzymatic reactions are the following:

1) Bacterial sphingomyelinase hydrolyzes SM and liberates phosphorylcholine plus n-acylsphingosine.

2) Alkaline phosphatase removes phosphate from phosphorylcholine and generates choline.

3) Catalyzed by choline oxidase, two molecules of choline together with two molecules of oxygen release two molecules of hydrogen peroxide.

4) Using peroxidase as a catalyst, two molecules of hydrogen peroxide together with one molecule of phenol and one molecule of 4-aminoantipyrine generates a red quinone pigment, which has optimal absorption at 505nm.

The standard curve is prepared from a known amount of choline dissolved in saline buffer.

Figure 2. The specificity determination of the SM enzymatic measurement. Using plasma sphingomyelin assay, a standard solution of SM generated a linear response over a wide concentration range. Then, SM and PC were mixed at different concentration in a 1:1 ratio and using the enzymatic method generated a standard curve. This curve is superimposed on the standard curve using SM alone indicating that there was no influence of PC on SM measurement.

Figure 3. The linearity of plasma SM enzymatic measurement. To test the linear range of plasma SM measurement, 1-10 ml human plasma was used to measure

the SM concentration. As indicated in Fig. 2, the plasma SM measurement is lenear from 1-6 µl plasma.

Figure 4. The correlation between the enzymatic and classical methods for SM measurement. 60 human plasma samples were assayed by both the enzymatic assay and classical method (lipid extraction/thin layer chromatography/ lipid extraction/ phosphate content measurement) to measure SM concentration.

Figure 5 The distribution of absolute concentration of plasma sphingomyelin values in cases and controls (SM/(SM+PC)).

Figure 6 The distribution of relative concentration of plasma sphingomyelin in cases and controls (SM/(SM+PC)).

Figure 7 Shows the relative increase in postprandial sphingomyelin concentration in plasma over a ten hour time interval.

Figure 8 Shows the structures of sphingosine (sphinganine) I-phosphate and its degradation products and the pathway for sphingolipid turnover. Key enzymatic steps and the consequences of their inhibition on the biosynthesis and intracellular levels of intermediates and end products in the turnover pathway are summarized. Both free sphingoid bases can be either reacylated in the endoplasmic reticulum or phosphorylated in the cytosol.

Figure 9A Shows the effect of Myriocin on cellular sphingomyelin synthesis in J774 cells.

Figure 9B Shows the effect of Myriocin on sphingomyelin secretion in J774 cell medium.

Figure 10A Shows the effect of Myriocin on cellular sphingomyelin synthesis in HepG2 cells.

Figure 10B Shows the effect of Myriocin on sphingomyelin secretion in HepG2 cell medium.

Detailed Description of the Invention

This disclosure provides a method for determining sphingomyelin concentration in a plasma or a tissue comprising the steps of:

(a) measuring an absorption spectrum of the plasma or tissue; and

(b) calculating the concentration of sphingomyelin from said measured absorption spectrum using calibration coefficients determined from a calibration set comprising absorbency spectra wherein the spectra of the reporter or molecule of said calibration set are varied by concentration,

thereby determining sphingomyelin concentration in the plasma or the tissue.

The absorption spectrum may be measured in a wavelength appropriate for the peak absorption of the selected reporter molecule, and may be in the visible wavelength region. The absorption spectrum may also be in the ultra-violet visible wavelength region.

The absorption spectrum may be measured utilizing a radioactive tracer as a detector substance.

This disclosure also provides a method for measuring an amount of sphingomyelin in a sample comprising the steps of:

(a) treating the sample containing sphingomyelin with a sphingomyelinase to produce phosphorylcholine and N-acylsphingosine;

(b) adding a phosphatase to the phosphorylcholine produced in step (a) to remove phosphate and generate a choline;

(c) reacting the choline of step (b) with suitable catalysts and suitable agents to form a chromogen; and

(d) measuring the optical density of the chromogen produced in step (c);

wherein the amount of sphingomyelin is determined by comparing the optical density obtained in step (d) with an optical density of a standard solution of sphingomyelin which generated a linear response over a concentration range.

The sample in step (a) may be plasma or tissue sample.

The sphingomyelinase in step (a) is selected from a group consisting of mammalian, eukaryotic, and bacterial sphingomyelinase. Preferably the sphingomyelinase in step (a) is bacterial sphingomyelinase.

The phosphatase in step (b) is selected from a group consisting of bacterial, eukaryotic, and alkaline phosphatase. Preferably the phosphatase in step (b) is alkaline phosphatase.

The suitable catalysts in step (c) may be choline oxidase or a peroxidase.

The method may be conducted in an oxygen containing atmosphere, wherein the suitable agents in step (c) is oxygen, 4-aminoantipyrine, and phenol.

The chromogen produced in step (c) may be red quinone pigment.

This disclosure further provides a method for measuring an amount of sphingomyelin in a sample comprising the steps of:

(a) treating the sample containing sphingomyelin with a sphingomyelinase to produce phosphorylcholine and N-acylsphingosine;

(b) adding a phosphatase to the phosphorylcholine produced in step (a) to remove phosphate and generate a choline;

(c) adding a first suitable catalyst and oxygen to the choline produced in step (b) to generate hydrogen peroxide;

(d) adding a second suitable catalyst, a 4-aminoantipyrine, and phenol to the hydrogen peroxide produced in step (c) to generate a chromogen; and

(e) measuring the optical density of the chromogen produced in step (d);

wherein the amount of sphingomyelin of step (a) is determined by comparing the optical density obtained in step (e) with an optical density of a standard solution of sphingomyelin which generated a linear response over a concentration range.

The sample in step (a) is plasma or tissue sample. The sphingomyelinase in step (a) is selected from a group comprising mammalian, eukaryotic, and bacterial sphingomyelinase.

The phosphatase in step (b) is selected from a group comprising bacterial, eukaryotic, and alkaline phosphatase.

The first suitable catalyst in step (c) may be choline oxidase.

The second suitable catalyst in step (d) may be peroxidase.

The chromogen of step (d) is red quinone pigment.

The optical density may be between 480 - 510nm, preferably optical density is between 490-505nm.

This disclosure yet further provides a method for measuring an amount of sphingomyelin in a sample comprising the steps of:

(a) treating the sample containing sphingomyelin with a bacterial sphingomyelinase to produce phosphorylcholine and N-acylsphingosine;

(b) adding an alkaline phosphatase to the phosphorylcholine produced in step (a) to remove phosphate and generate a choline;

(c) adding a choline oxidase and oxygen to the choline produced in step (b) to generate hydrogen peroxide;

(d) adding a peroxidase, a 4-aminoantipyrine, and phenol to the hydrogen peroxide produced in step (c) to generate a red quinone pigment; and

(e) measuring the optical density of the red quinone pigment produced in step (d);

wherein the amount of sphingomyelin of step (a) is determined by comparing the optical density obtained in step (e) with an optical density of a standard solution of sphingomyelin which generated a linear response over a concentration range.

This disclosure also provides a method for determining whether a compound inhibits sphingomyelin biosynthesis or induces sphingomyelin clearance comprising the steps of:

(a) culturing cells which produce and secrete sphingomyelin in culture;

(b) measuring the sphingomyelin level in the culture according to the method of claim 1;

(b) administering the compound to be tested to the culture;

(c) measuring sphingomyelin level in the culture at various intervals of time after step (b) using the method of claim 1; and

(d) comparing the sphingomyelin level obtained in step (c) with the sphingomyelin level obtained in step (a), wherein the compound administered in step (b) inhibits sphingomyelin biosynthesis or induces sphingomyelin clearance if the sphingomyelin level measured in step (c) is lower than the sphingomyelin level measured in step (a).

In this method, the cells may be liver cells.

This invention finally provides a method of treating an atherosclerotic disorder in a subject which comprises administering to the subject a pharmaceutical composition comprising an effective amount of a compound that reduces plasma sphingomyelin concentration.

The compound may inhibit sphingomyelin biosynthesis or induce sphingomyelin clearance.

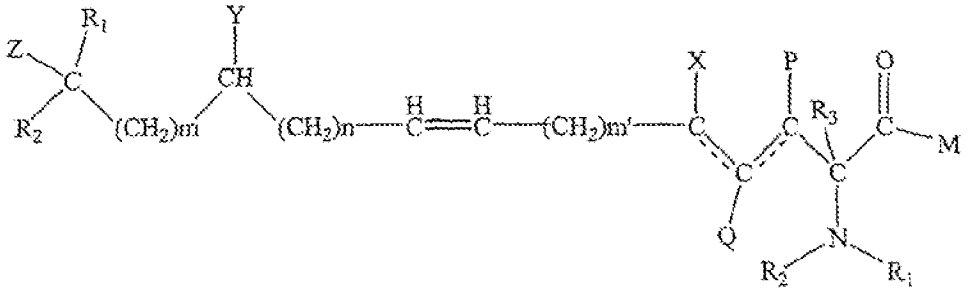
The atherosclerotic disorder may be coronary heart disease, hyperlipidemia, hypertriglyceridemia, familial hypercholesterolemia, atherosclerosis, or a renin/angiotensin control disorder.

The compound may be selected form the group consisting of ISP-1/myriocin, sphingofungin C, lipoxamycin, haloalanines, cycloserine, fumonisin B1, AAL-toxin, and australigungin.

The compound may be a serine palmitoyltransferase inhibitor, a ceramide synthase inhibitor, a cerebroside synthase inhibitor, a shphingosine kinase inhibitor, and a ceramidase inhibitor.

Preferably, the compound inhibits serine palmitoyltransferase.

In this method the compound has the structure:



wherein each of R_1 and R_2 is the same or different and is hydrogen, or a C_1-C_4 substituted or unsubstituted hydrocarbon;

wherein R_3 is benzoyl or a halogen substituted benzoyl;

wherein Z is halogen, hydroxyl, amino, or C_1-C_4 substituted or unsubstituted hydrocarbon;

wherein each of m and m' is the same or different and is either 0 or 1, such that when m or m' is 0 then the respective (CH_2) group is absent;

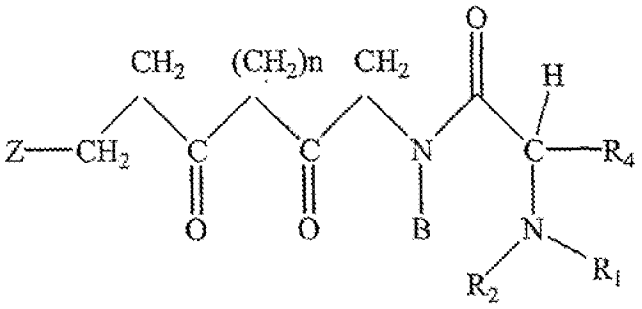
wherein n is an integer between 1 and 18;

wherein each of P, Q, X and Y is the same or different and is halogen, amino, or C_1-C_4 substituted or unsubstituted hydrocarbon;

wherein M is hydroxyl, amino, or C_1-C_4 substituted or unsubstituted hydrocarbon; and

wherein the dashed line represents a covalent bond that is either present or absent.

In this method the compound may also have the structure:



wherein each of R_1 and R_2 is the same or different and is hydrogen, or a C_1-C_4 substituted or unsubstituted hydrocarbon;

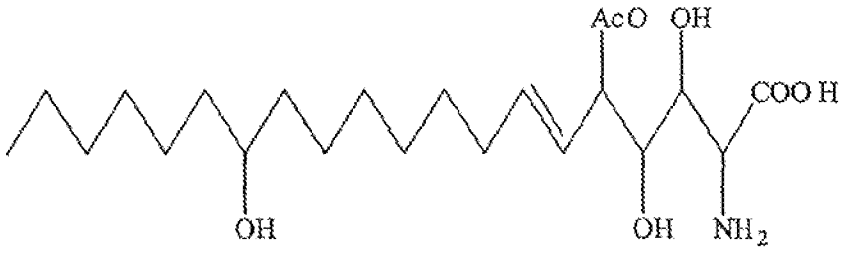
wherein R_4 is hydroxyl, amino, or C_1-C_4 substituted or unsubstituted hydrocarbon;

wherein Z is halogen, hydroxyl, amino, or C_1-C_4 substituted or unsubstituted hydrocarbon;

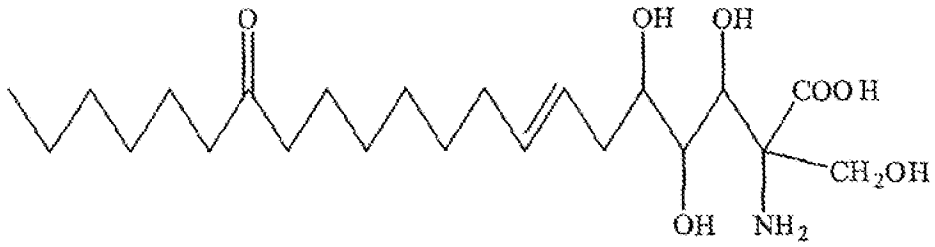
wherein n is an integer between 1 and 18; and

wherein B is halogen, amino, or C_1-C_4 substituted or unsubstituted hydrocarbon.

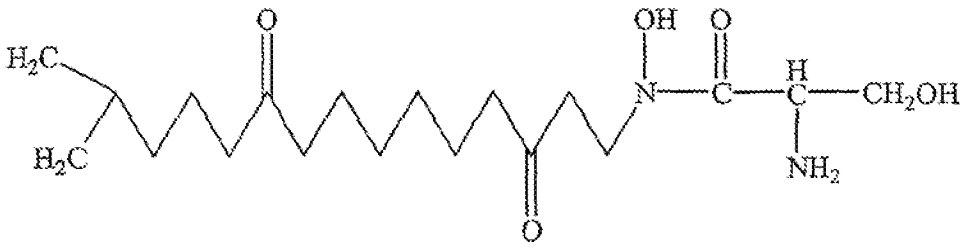
The compound may be:



The compound may also be:



The compound may also be:



Thus, this invention is a new enzymatic method of plasma and tissue sphingomyelin measurement which differs from the closest prior art in that 1) it is not time-consuming and laborious; 2) it needs only 5 μl or less, instead of several hundred μls of plasma; 3) large scale samples (hundreds) can be measured quickly (within one hour); and 4) it is readily amenable to automation. The invention includes four simple enzymatic reactions leading to plasma SM measurement. Plasma SM levels obtained by the new method are well correlated ($r \approx 0.9$, $p < 0.01$) with those using the classical method (lipid extraction thin layer chromatography, elution and phosphate determination).

This invention also demonstrates that human plasma sphingomyelin levels are strongly positively correlated with atherosclerosis and coronary heart disease. In the case and control study herein, results show that plasma sphingomyelin concentration in coronary heart disease patients are significantly higher than that of controls without disease, and this relationship with the disease is independent from plasma cholesterol and LDL cholesterol levels. The use of a quick and effective plasma sphingomyelin measurement such as the subject invention, is valuable as a diagnostic test, for screening assays in vitro, in cell culture or in animals to develop drugs or other treatments aimed to lower plasma sphingomyelin levels. The findings indicate that therapies aimed at reducing plasma or tissue SM levels are likely to have therapeutic benefit. These would include inhibition of sphingomyelin synthesis in the liver or arterial wall, as well as methods to enhance clearance of sphingomyelin from plasma. Any approach which leads to decrease sphingomyelin concentration in plasma is considered an antiatherogenic measurement.

Furthermore, this invention predicts coronary heart disease better than plasma cholesterol or LDL cholesterol measurement. Studies performed herein show that plasma sphingomyelin measurement have better predictive value for coronary heart disease than plasma cholesterol or LDL cholesterol measurement.

The following Experimental Details are set forth to aid in an understanding of the invention, and are not intended, and should not be construed, to limit in any way the invention set forth in the claims which follow thereafter.

Experimental Details

Plasma Sphingomyelin Assay

The plasma sphingomyelin assay begins with four sequential enzymatic reactions leading to the production of a pigment which is then measured. The four sequential enzymatic reactions are the following:

1) Bacterial sphingomyelinase hydrolyzes SM and liberates phosphorylcholine plus n-acylsphingosine.
2) Alkaline phosphatase removes phosphate from phosphorylcholine and generates choline.
3) Catalyzed by choline oxidase, two molecules of choline together with two molecules of oxygen release two molecules of hydrogen peroxide.
4) Using peroxidase as a catalyst, two molecules of hydrogen peroxide together with one molecule of phenol and one molecule of 4-aminoantipyrine generates a red quinone pigment, which has optimal absorption at 505nm.

Enzymatic Sphingomyelin Measurement

A. Color Reagent: Bacterial sphingomyelinase (10U), alkaline phosphatase (500U), choline oxidase (25U), peroxidase (2500U) and 7.5 mg 4-aminoantipyrine was dissolved into a 50 ml buffer (0.05M Tris-HCl, pH 8.0, 5mg/dl $CaCl_2$ and 0.05% phenol).

B. Assay:

1) *Standard curve*: A sphingomyelin (Sigma) stock solution (300 mg/dl in ethanol) was prepared. 0ml, 1ml, 2ml, 3ml, 4ml, and 5ml of the stock solution were taken to each well (in 96-well plate) and ddH_2O was supplemented to 5ml, then, 120ml color reagent was added. The mixture was incubated at 37°C for 30 min. and the pigment was assessed by measuring the optical density (OD) at 492nm on 96-well reader (TECAN).

While the optimal reading is at 505 nm, some of the OD readers used (the "old type" 96-well OD readers) can only read the 490 nm and 492 nm wavelengths. Despite this, the sphingomyelin measurement can still be performed using these machines as long as the standard curve and the samples are assayed using the same machine or calibration.

2) *Plasma sample assay*: Five ml of plasma and 120ml of color reagent were incubated on 96-well plate at 37°C for 30 min and the pigment was assessed by measuring the OD at 492nm on 96-well reader (TECAN). Based on the standard curve, the SM concentration can be calculated. Total plasma phospholipids (SM and PC) were assayed by an enzymatic Kit (Wako Pure Chemical Industries Ltd., Osaka, Japan). PC concentration was obtained by subtracting SM from total phospholipids.

3) *Tissue sample assay*: Thirty ml of chloroform/methanol (2:1) was mixed with 0.5 gram of tissue which was homogenized with 2 ml ddH_2O. The mixture was shaken at room temperature for 30 min, then, 6 ml 0.05% of H_2SO_4 was added and aqueous and organic phases were separated by centrifugation at 2000 rpm. To the 20 ml organic phase, 2 ml 10% Triton X-100 was added and 600 ml of it was dried down under N_2 gas. The lipids were suspended by 300 ml 2% Triton X-100 in PBS solution. Tissue SM concentration can be assayed using 5 µl of above lipid suspension and 120 µl color reagent.

Classical Method for SM and PC Measurement

Lipid extracts (37) of plasma were separated by TLC on silica gel (Adsorbosil plus; Alltech Associates, Inc., Deerfield, IL) using chloroform/methanol/acetic acid/H_2O (50:25:8:4; vol/vol/vol/vol). Individual

phospholipid subclasses were identified by comparison with standards. The spots were scraped, extracted twice with chloroform/methanol/H_2O (5:10:4; vol/vol/vol), and assayed for phosphate content by the method of Bartlett (38).

Example 1

The Study Population

To assess the relationship between plasma SM levels and coronary heart disease (CHD), a novel, high throughput, enzymatic method to measure plasma SM levels has been developed. Plasma SM levels were related to presence of CHD in a biethnic angiographic case-control study [cases, n=279 (Whites 181, Blacks 98); controls n=277 (Whites 146, Blacks 131)].

Subjects were recruited from a patient population scheduled for diagnostic coronary arteriography at either Harlem Hospital Center in New York City or the Mary Imogene Bassett Hospital in Cooperstown, NY. All consecutive patients scheduled for coronary arteriography at the two sites between June 1993 and April 1997 were approached. A total of 628 patients, 341 men and 215 women, ethnically self-identified as African Americans (n=229), Caucasians (n=327) or Other (n=72) were enrolled. Due to the small number of subjects ethnically identified as Other (mostly Hispanics), the present report is based on the findings in the 556 African Americans and Caucasians. Mean age was 54.6 years old for African American men and women, 56.8 and 56.5 years old for Caucasian men and women, respectively. Exclusion criteria were: age > 70 years, recent (within 6 months) myocardial infarction or thrombolysis, a history of percutaneous transluminal coronary angioplasty (PTCA), surgery during the previous six weeks, a known communicable disease such as hepatitis or AIDS, or current lipid-

lowering medication. Information on diabetes mellitus, hypertension, and smoking was obtained by a standardized questionnaire upon entry into the study. Among Caucasians, 25.6%, 27.3% and 20.4% of the men and 20.5%, 25.6% and 20.3% of the women were smokers, had hypertension and diabetes, respectively. The corresponding numbers for African Americans were 51.1%, 67.4% and 24.2% for men, and 42.0%, 78.2% and 35.6% for women. The study was approved by the Institutional Review Boards at Harlem Hospital, Bassett Healthcare, and Columbia University College of Physicians and Surgeons.

Plasma Sphingomyelin Measurement

Enzymatic measurement of plasma sphingomyelin levels was carried out using a novel four step procedure. In the first step, bacterial sphingomyelinase hydrolyzed sphingomyelin to phosphorylcholine and n-acylsphingosine. Thereafter, addition of alkaline phosphatase generated choline from phosphorylcholine. The newly formed choline was used to generate hydrogen peroxide in a reaction catalyzed by choline oxidase. Finally, using peroxidase as a catalyst, hydrogen peroxide was used together with phenol and 4-aminoantipyrine to generate a red quinone pigment, with an optimal absorption at 505nm.

The plasma sphingomyelin levels were measured in a blinded fashion. The linear range of plasma SM in this assay was between 10 μg/dl and 120 μg/dl. The interassay CV(%) of the SM assay was 2.8$\pm$0.3%. There was no influence of PC on the SM measurement. To validate the subject novel SM assay, the results were compared with those obtained by the classical method (29,30). The two methods were well correlated (r=0.91, p<0.01, n=60).

Plasma phosphatidylcholine (PC) Measurement

The total choline-containing phospholipid in plasma was assayed by enzymatic method (Wako Pure Chemical Industries Ltd., Osaka, Japan). PC concentration was obtained by substrating SM from total phospholipid concentration.

Angiographic Definition of Coronary Artery Disease

Coronary angiograms were read by two experienced readers, blinded to patient identity, the clinical diagnosis and the lipoprotein results. The readers recorded the location and extent of luminal narrowing for 15 segments of the major coronary arteries (31). Presence of CAD (i.e. case) was defined as the presence of at least 50% stenosis in any one of 15 coronary artery segments.

Statistical Analysie

Comparison of means between groups were made using the Wilcoxon test. Fisher's Exact test was used to calculate p-values for the odds ratios (OR) of the association of univariate categorical data with case-control status. Conditional logistic regression was used to assess association with case-control status for multivariate models. SAS was used for all calculations.

Results and Discussion of Example 1

Specificity Determination of the SM enzymatic measurement

Using the plasma sphingomyelin assay, a standard solution of SM generated a linear response over a wide concentration range. Since the assay is based on generation and measurement of choline, possible interference from other choline-containing lipids was

assessed. Thus SM and PC were mixed at different concentration in a 1:1 and the method was used to measure SM in the SM/PC mixture or in a sample of pure SM. Results in **Figure 2**, show that the SM and SM/PC curves are superimposed, indicating that there was no influence of PC on the SM measurement.

Consistency of Plasma SM Enzymatic Measurement

To test the 1μ linear range of plasma SM measurement, human plasma from 1-10 μl were taken to measure the SM concentration. As indicated in **Figure 3**, the linearity of plasma SM measurement can reach within 1-6 μl plasma. Five μl plasma was chosen to do the rest of the experiments.

In order to effectively measure SM in plasma and tissues, consistency of the subject method must be proven. The precision of the SM measurement was established by analyzing three human plasma samples and three liver samples in replicate (totaling 12 sample measurements). The CV(%) (% coefficient of a variation) of these measurements was 2.78±0.29% and 3.69±0.38%, respectively, suggesting that the precision of this method was high. The accuracy of the measurement was determined by adding exogenous SM into three human plasma samples: 6 μl of 20mg/dl SM (in ethanol) was added to 500 μl plasma and the final SM concentration should be the endogenous SM concentration plus 24 mg/dl of exogenous SM. After subtracting the endogenous SM value, the exogenous SM was estimated to be 23.66±2.88 mg/dl (n=6), in good agreement with the expected value of 24 mg/dl.

Precision of SM measurement in Liver Lipid Extracts

The precision of the SM Measurement in Liver Lipid extracts was done by analyzing three liver samples in replicate. The results are shown in Table 1.

Liver Lipid Extraction and SM assay

1) 0.5g Liver + 2 ml. PBS, homogenization, then add 30 ml. of $CHCl_3$:MeOH (2:1), shaking, 30 min at room temperature, then add 6 ml. 0.5% H_2SO_4 to separate two phases.

2) Collect bottom phase (organic) and record the Vol.

3) Store 20 ml. organic phase under N_2.

4) Take 1 ml. organic phase and add 1 ml. of 1% Triton X 100 (in $CHCl_3$).

5) Dry down the $CHCl_3$.

6) Add 0.5 ml. dd H_2O, 1.5 min. at 37°C.

7) Using 50 µl. above solution, add 120 µl. SM color reagent for SM assay.

8) For Standard Curve: take 2 µl., 4 µl., 8 µl., and 10 µl. of Standard Solution and add ddH_2O to 50 µl., then add 120 µl. color reagent.

Table 1

Replicate No.	Sample 1 OD 505	Sample 2 OD 505	Sample 3 OD 505
1	0.438	0.357	0.302
2	0.505	0.362	0.318
3	0.472	0.385	0.309
4	0.462	0.355	0.292
5	0.483	0.352	0.314
6	0.472	0.366	0.315
7	0.477	0.341	0.303
8	0.481	0.333	0.299
9	0.492	0.351	0.300
10	0.466	0.368	0.309
11	0.461	0.377	0.310
12	0.478	0.334	0.302
$\bar{x}$	**0.473**	**0.356**	**0.306**
SD	0.0168	0.0161	0.0092
CV%	**3.55**	**4.52**	**3.00**

Example 2

Plasma SM Measurements by Enzymatic Assay Compared to Classical Method

To further evaluate the application of this method for measuring plasma SM concentrations, 60 human plasma samples were randomly chosen from the case and control study which will be discussed below and tested using both new method and the classical method (lipid extraction/thin layer chromatography/lipid extraction/phosphate content measurement) to measure SM concentration. Results of the test show that the new method is well correlated with the classical method ($r=0.9$, $p<0.01$) (**Figure 4**). The enzymatic method gave a SM concentration of 52 ± 16 mg/dl, while the TLC method gave a SM concentration of 45 ± 13 mg/dl. The value obtained by TLC method was assessed by adding radiolabeled SM into plasma as an internal standard. This showed that recovery was incomplete, explaining the consistently lower estimation of SM by the classical method.

Plasma SM Measurement by Enzymatic Assay as an Independent Risk Factor for Coronary Heart Disease

For all subjects, patients with coronary artery disease (CHD) had significantly higher mean plasma SM concentrations than controls (60 ± 29 vs. 49 ± 21 mg/dl, $p<0.0001$). (Table 2). When analyzing the two ethnic groups separately, the plasma SM concentration was significantly increased in both African Americans and Caucasians with CHD ($p<0.0001$ and $p=0.012$, respectively, Table 2). As seen in Figures 5 and 6, the distribution of plasma SM was skewed in both cases and controls. However, the tendency for CHD cases to have higher SM levels than controls was seen over the entire range of SM values (Fig. 5). As expected from the skewed distribution, median SM levels were lower than mean levels in both ethnic groups (Table 2), but

remained significantly increased in cases compared to controls (Table 2).

In order to evaluate whether the increased plasma SM levels among cases reflected an overall increase in phospholipid levels or an increased proportion of SM amongst total plasma choline-containing phospholipids, a study was performed comparing the SM/(SM+PC) ratio (relative concentration of SM) in case and control groups. The SM/(SM+PC) ratio of cases was significantly higher than controls among all subjects (0.33±0.13 vs. 29±0.10, p<0.0001), as well as among African Americans and Caucasians when the two groups were analyzed separately (Table 2). However, the difference in the SM/(SM+PC) ratio (about 14%) between cases and controls was smaller than the difference in total SM (about 22%), indicating that the ratio only partly accounted for the increase in plasma SM concentrations. Figure 6 shows the SM/(SM+PC) ratio distribution in all subjects. Again, the distribution was skewed and median SM/(SM+PC) ratio were lower than mean SM/(SM+PC) ratio in both ethnic groups (Table 2).

Table 2. SM Concentration and SM/(SM+PC) Ratio in Case and Control Samples

	n	SM (mg/dl)	Median	IQR*	pValue**	SM/(SM+PC)	Median	IQR*	pValue**
All									
Control	277	49±21	44	24		0.29±0.10	0.27	0.12	
Case	279	60±29	52	21	<0.0001	0.33±0.13	0.30	0.13	<0.0001
Whites									
Control	146	50±22	45	20		0.29±0.11	0.27	0.13	
Case	181	63±31	54	26	<0.0001	0.33±0.14	0.30	0.14	0.0026
Blacks									
Control	131	48±20	43	22		0.28±0.09	0.27	0.10	
Case	98	55±23	50	22	0.0118	0.32±0.10	0.31	0.12	0.0006

*IQR, interquartile range. ** Wilcoxon test

To evaluate whether the plasma SM concentration was associated with case-control status, odds ratios (OR) were calculated based on univariate logistic regression analysis. Since African Americans and Caucasians had similar mean and median values for both the absolute and relative concentration of SM, all subjects were grouped together in this analysis. Subjects were divided into quartiles for both SM levels and SM/(SM+PC) ratio. After adjusting for other risk factors, the OR for CHD was increased from the first to the fourth quartiles. The OR for CHD for the third and fourth quartiles was significantly higher than the first quartile for both measurements (Table 3).

Table 3 Odds ratio (OR) based on univariate logistic regression analysis.

Quartile	Control (n)	Case (n)	p Value	Odds Ratio	Lower CI*	Upper CI*
SM						
1	89	50	--	1	--	--
2	75	64	0.33	1.30	0.80	2.11
3	59	80	<0.0001	2.83	1.74	4.60
4	54	85	0.0001	2.59	1.60	4.19
SM/(SM+PC)						
1	89	50	--	1	--	--
2	75	64	0.11	1.52	0.94	2.46
3	59	80	0.0005	2.41	1.49	3.91
4	54	85	0.0004	2.80	1.72	4.56

*CI, Confidence Interval.

To evaluate whether the plasma concentration of SM and the SM/(SM+PC) ratio was associated with CHD independent of other known risk factors, a multivariate logistic regression was calculated controlling for age, diabetes, smoking, hypertension, LDL-C, HDL-C, log-transformed TG, apoB, fibrinogen and C-reactive protein. The OR for CHD increased with

increasing quartiles of both SM levels and SM/(SM+PC) ratio. As shown in Table 4, the OR for CHD in the third and fourth quartiles of SM levels was significantly higher than in the first quartile (p=0.0001 and p=0.0017, respectively), indicating that plasma SM levels was an independent risk factor for CHD in this case-control study. In addition, the OR for CHD for the relative concentration of SM, i.e. SM/(SM+PC) ratio, was significantly higher for the third and fourth quartiles compared to the first quartile (p=0.014 and p=0.0017, respectively), indicating that the relative concentration of SM was also associated with CHD, independent of age, diabetes, smoking, hypertension, LDL-C, HDL-C, log-transformed TG, apoB, fibrinogen and C-reactive protein.

Table 4. Multivariate results from stepwise logistic regression controlling for age, diabetes, smoking, hypertension, LDL-C, HDL-C, apoB, Log-transformed TG, fibrinogen and C-reactive Protein.

	Quartile	Odds Ratio	p Value	Lower CI*	Upper CI*
SM					
	1				
	2		NS		
	3	2.85	0.0001	1.67	4.85
	4	2.30	0.0017	1.37	3.86
SM/(SM+PC)					
	1				
	2		NS		
	3	2.03	0.014	1.15	3.58
	4	2.42	0.0017	1.39	4.19

*CI, Confidence Interval

Conclusion. The findings indicate that human plasma SM levels are positively and independently related to coronary heart disease in African Americans and Caucasians.

Results and Discussion of Example 2

Uses for an Efficient Enzymatic Measurement of Plasma Sphingomyelin Concentration

Although traditional measurements have focused on plasma total and LDL cholesterol as indicators of atherogenesis, a body of *in vitro* and *in vivo* results (14, 18, 23-27) have suggested that plasma SM levels could also be related to atherosclerosis. However, this has never been systematically assessed, partly because of the difficulties of measuring SM in large number of samples. To overcome this problem, a simple enzymatic assay was developed to permit measurement of SM concentration in a large number of plasma samples. In the present study, measurements indicate that plasma SM levels were higher in cases with CHD than controls, and this difference was found both for African Americans and Caucasians. Moreover, SM measurements may be more strongly and independently associated with case-control studies than that of LDL-C and HDL-C measurements. The increase in plasma SM was selective, reflected as an increase in SM concentration relative to other phospholipids (SM/(SM+PC) ratio), and the relative SM concentration was also independently related to CHD case-control status. These findings are biologically plausible (12,13).

A number of different mechanisms could explain the relationship between plasma SM and CHD case-control status. Since LDL is an atherogenic lipoprotein, SM may be a surrogate marker for LDL cholesterol levels. However, this appears unlikely as the SM relationship to case-control status was independent of LDL-C levels (Table 4). SM could also be a marker for an inflammatory effect, and inflammatory markers such as C-reactive Protein (CRP) have been shown to be important risk factors for atherosclerosis (39).

However, in this case-control study, plasma SM levels did not correlate with two well-known inflammatory markers, fibrinogen and CRP, and were independently related to case-control status in a multivariate analysis which included these measurements (Table 4). Thus, it is unlikely that SM is behaving as a surrogate inflammatory marker.

The hypothesis through experiments performed is that plasma SM levels are determined by a unique set of metabolic determinants, and that plasma SM, carried by lipoproteins, is directly involved in the atherogenic process subsequent to retention in the artery wall (12,28,40,41). Thus, the conclusion drawn is that plasma SM levels are directly and causally related to atherogenesis.

Substantial evidence now supports the role of lipoprotein sphingomyelin and arterial sphingomyelinase (SMase) in atherogenesis. Sphingomyelin carried into the arterial wall on atherogenic lipoproteins is acted on by an arterial wall sphingomyelinase, leading to an increase in ceramide content and promoting lipoprotein aggregation (12). LDL extracted from human atherosclerotic lesions is highly enriched in SM compared to plasma LDL (12,35,36). Moreover, a significant fraction of LDL extracted from fresh human lesions is aggregated and has a high content of ceramide, indicating that the LDL has been modified by SMase, resulting in aggregation (12). This degree of LDL modification is sufficient to induce macrophage foam cell formation. A leading candidate for the arterial wall enzyme is a Zn^{2+}-dependent SMase that is secreted by cultured macrophages (40) and endothelial cells (40), both of which are major cellular constituents of atherosclerotic lesions. The activity of this enzyme

may be induced by cytokines known to be present in lesions, such as IL-1β and interferon-γ (42,43). The secreted SMase is derived from the same gene and mRNA as that encoding lysosomal SMase. Both the absolute and relative concentrations of plasma SM are increased in atherosclerosis susceptible rabbits(25), monkeys (26) and apoE knock-out mice (27). In the latter model this has been shown to markedly increase susceptibility to secretory SMase (27). In vitro manipulation has shown that the relative SM concentration is an important determinant of susceptibility to SMase-induced aggregation (27,28). Recently, transgenic animals with increased or decreased SMase activity in the arterial wall have been shown to have correspondingly altered atherosclerosis (44).

Plasma lipoprotein SM is derived principally from biosynthesis in the liver. The rate-limiting step in SM biosynthesis is the enzyme serine:palmitoyl CoA transferase (SPT). The increase in plasma SM concentration in apoE knock-out mice partly reflects increased activity of this enzyme (27). The synthesis and metabolism of plasma SM is distinct from that of cholesterol and PC. Inhibitors of SPT have been described, so there might be some potential for therapeutic modulation, at the level of hepatic synthesis. Alternatively the arterial wall SMase could represent another target for intervention.

Unlike plasma PC, SM is not degraded by plasma enzymes such as lecithin cholesterol acyltransferase (LCAT) or by lipases (45,46). Thus, SM removal from plasma is absolutely dependent on hepatic clearance mechanisms, such as the LDL receptor, the LDL receptor related protein or proteoglycan pathways. Clearance is facilitated by apoE, as shown by the delayed clearance

of SM in apoE knock-out mice (27). Since SM is not degraded in plasma, it tends to become enriched in atherogenic remnants of triglyceride-rich lipoproteins (25,27). Several lines of evidence suggest that remnants of triglyceride rich lipoproteins are particularly atherogenic (47), but the relevant fraction of plasma lipoproteins has been difficult to measure. In part plasma SM measurements appear to be acting as a marker of atherogenic remnant accumulation.

Although presently known risk factors have some predictive value for CHD, a major part of the variability in this process remains unexplained (48). Also, therapy aimed at lowering LDL cholesterol only reduces a fraction (roughly 30%) of the burden of atherosclerotic disease (49). While our findings that SM is a risk factor for CHD needs to be confirmed in additional studies, they hold the promise of an simple test that may have independent predictive value for coronary artery disease.

SM could also be a marker for the inflammatory effect which is considered one of the risk factors for atherosclerosis. Infection and inflammation induce a wide array of metabolic change, called the acute phase response, that protect the animal from further injury and helps in the repair response (50). The hepatic synthesis of certain protein, such as C reactive protein, serum amyloid A, and HMGCoA reductase (positive acute phase proteins), are increased, while the synthesis of other proteins, such as albumin, transferrin, and apoE (negative acute phase proteins) are inhibited (50,51). The acute phase response in rodents stimulates fatty acid and cholesterol (51) synthesis. Induced lipopolysaccharide (LPS) which increases in sphingolipids (including SM synthesis),

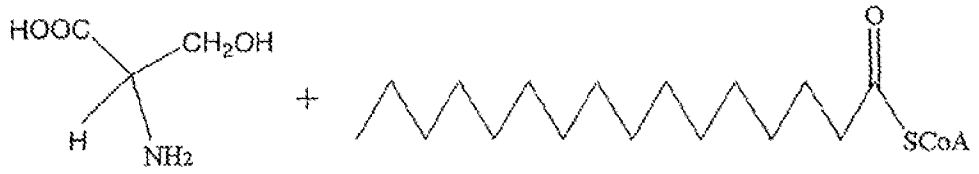
serine palmitoyltransferase (SPT) (a key enzyme for sphingolipid synthesis) activity and SPT mRNA levels in the liver can be considered part of the acute phase response (52), thus SPT is a positive acute phase protein. Inhibitors for serine palmitoyltransferase include ISP-1/myriocin, sphingofungin C, lipoxamycin, haloalanines and cycloserine. Inhibitors for ceramide synthase include fumonisin B1, AAL-toxin, and australigungin. Many of the metabolic changes that occur during the acute phase response are induced by cytokines, particularly TNF, IL-1 and IL-6 (53,54). These cytokines directly regulated SPT mRNA levels and sphingolipids synthesis in hepatocytes indicating, again, that SPT was linked to the acute phase response. Thus, SM may also be an inflammatory marker in the case and control study.

However, the main hypothesis is that plasma SM levels are determined by a unique set of metabolic determinants, and that plasma SM, carried by lipoproteins, is directly involved in the atherogenic process by deposition and retention in artery wall as a result of a local interaction with sphingomyelinase (55). The subendothelial retention and aggregation of SM-rich lipoproteins appear to be early events in atherogenesis (56). It is notable that SM has a unique biosynthetic pathway with completely different regulation to cholesterol biosynthesis (32). Clearance of SM may also be governed by a distinctive set of factors, including apoE and perhaps unknown receptors or enzymes in the liver (36). Evidence show that increased liver SPT activity in apoE knock-out mice was responsible for the SM enrichment in plasma. LDL isolated from these mice had a strong tendency to be aggregated after mammalian sphingomyelinase treatment at neutral pH (36). Inhibitors of SPT have been described (57) and the present results suggest

that these inhibitors could have antiatherogenic properties. Evidence also shows that the clearance of SM-enriched lipoproteins from apoE knock-out mice plasma, mainly through liver, was delayed by 50% (36). The clearance pathway is independent of LDL receptor and LDL receptor related protein (36). Thus, decreased plasma SM could be achieved by inhibiting synthesis or through stimulation of clearance. Any approaches which lead to decrease of plasma SM concentration in CHD patients could be antiatherogenic. The new method for SM measurement will be useful in development of such therapies.

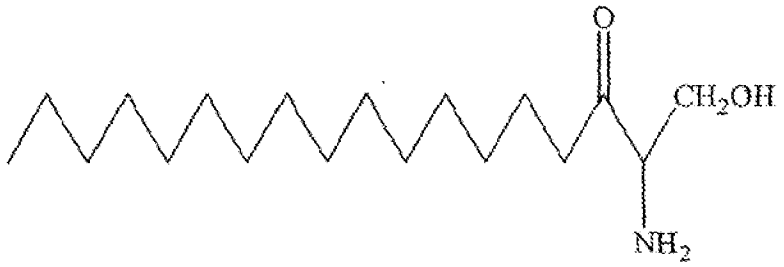
Example 3 - Inhibition of Sphingomyelin (SM) Synthesis

The following shows *de novo* sphingolipid biosynthesis. Key enzymatic steps and the consequences of their inhibition on the biosynthesis and intracellular levels of intermediates and end products in the *de novo* pathway are summarized. In mammalian cells, *de novo* sphingolipid biosynthesis begins in the endoplasmic reticulum and proceeds via the reactions shown with the headgroups on the 1 hydroxyl being added in the Golgi (Merrill et al., 1996c). Headgroups: sphingomyelin, R_1 = $OP(O_2H)OCH_2CH_2N(CH_3)_3$; examples of glycosphingolipids, R_1 = lactosylceramide = O-glucose-galactose, R_1 = gangliodide G_{M3} = O-glucose-galactose-sialic acid.

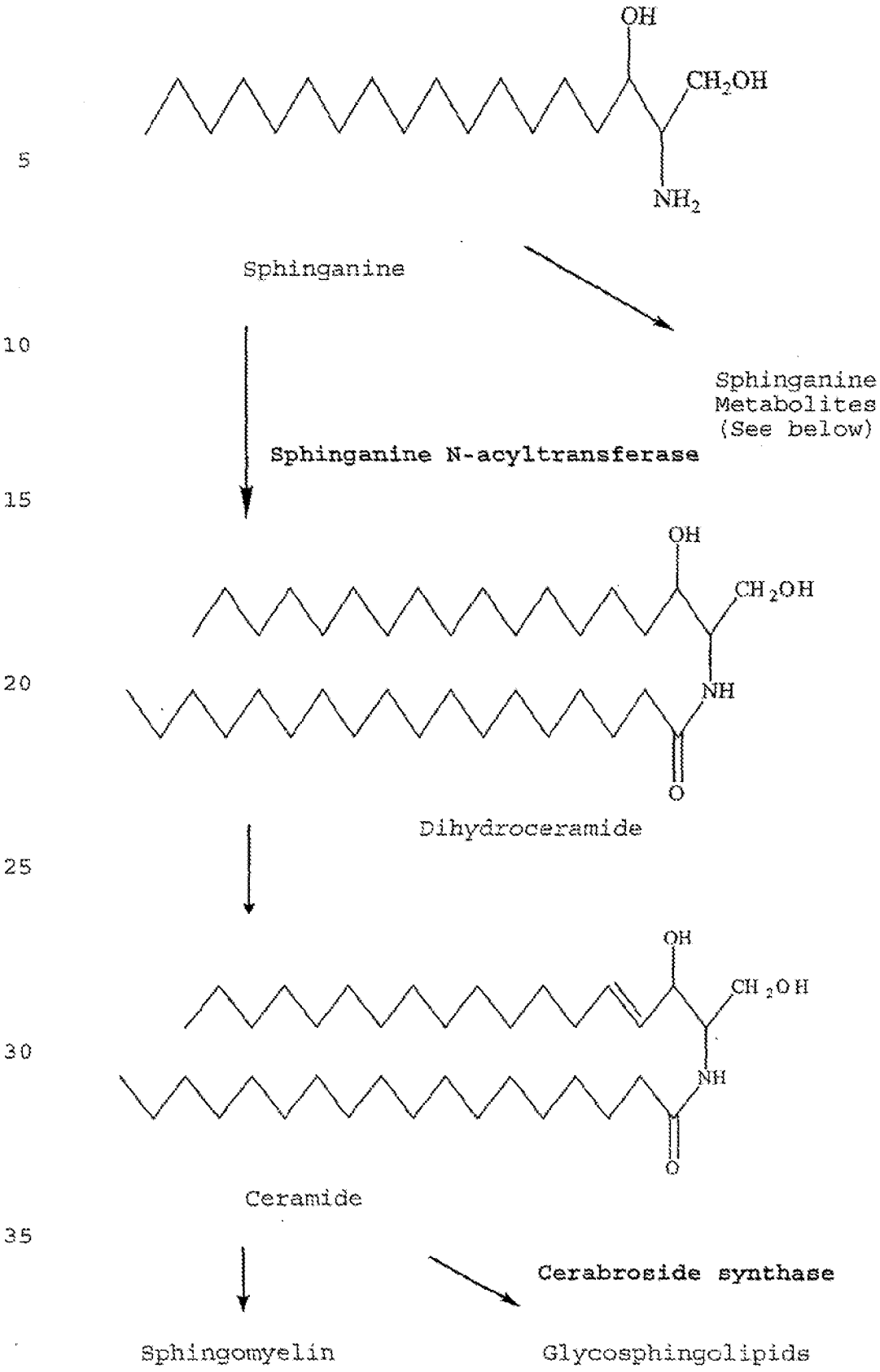


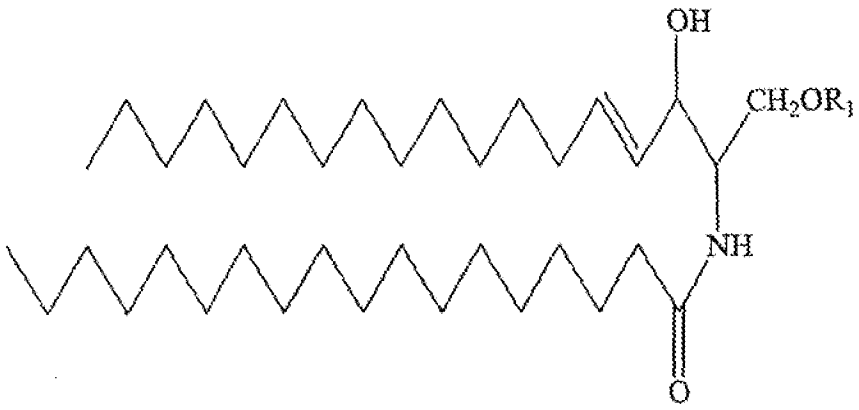
Serine Palmitoyl-CoA

Serine Palmitoyltransferease



3-Ketosphinganine


OH
CH2OH
NH2
Sphinganine
Sphinganine
Metabolites
(See below)
Sphinganine N-acyltransferase
OH
CH2OH
NH
O
Dihydroceramide
OH
CH2OH
NH
O
Ceramide
Cerabroside synthase
Sphingomyelin
Glycosphingolipids



Sphingomyelin (SM) - R_1 = $OP(O_2H)OCH_2CH_2N(CH_3)_3$;

examples of glycosphingolipids, R_1 = lactosylceramide = O-glucose-galactose, R_1 = ganglioside G_{M3} = O-glucose-galactose-sialic acid.

Sphinganine Metabolites

The structure of sphinganine metabolites referred to above and of sphingosine (sphinganine) I-phosphate and its degradation products and the pathway for sphingolipid turnover are shown in Figure 8. Key enzymatic steps and the consequences of their inhibition on the biosynthesis and intracellular levels of intermediates and end products in the turnover pathway are summarized. Both free sphingoid bases can be either reacylated in the endoplasmic reticulum or phosphorylated in the cytosol.

The descriptions of enzymes referred to in the above two schemes follow.

Serine palmitoyltransferase - Decreases biosynthesis of sphinanine, subsequent metabolites, and more complex shingolipids.

Sphinganin N-acyltransferase - Increases biosynthesis of sphinganine and its matabolites. Decreases biosynthesis of more complex sphingolipids.

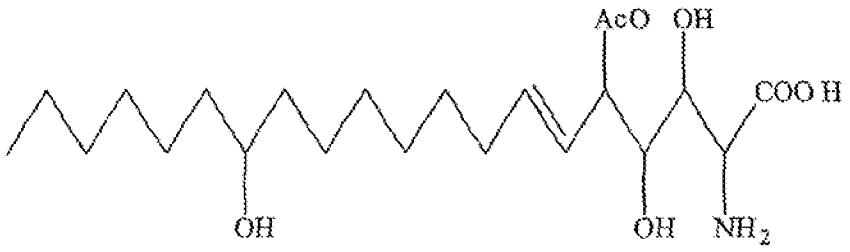
Cerabroside synthase - Decreases biosynthesis of glucosylceramide. Increases levels of sohingosine, ceramide and 1-O-acylceramide.

Ceramidase - Decreases sphingosine and sphingosine-1-P from ceramide turnover.

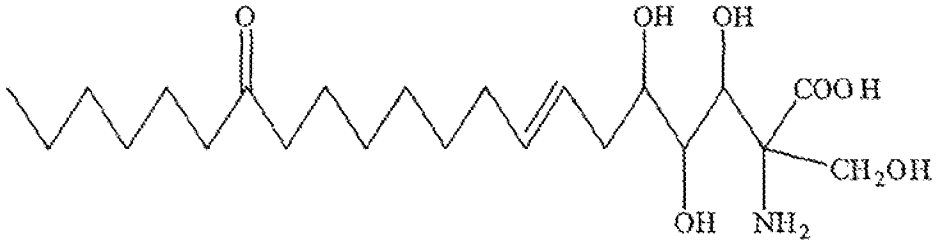
Sphingosine kinase - Decreases biosynthesis of sphinganine-1-P, sphingosine-1-P, ethanolamine-P, phosphatidylethanolamine, etc.

The following are natural and synthetic inhibitors of the certain enzymes in the *de novo* sphingolipid biosynthetic and turnover pathways.

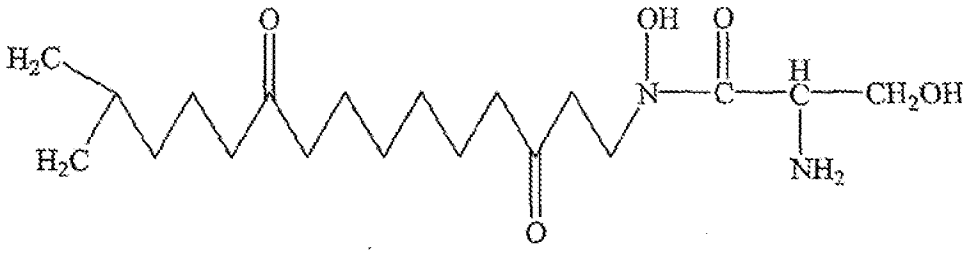
Serine Palmitoyltransferase Inhibitors



Sphingofungin C

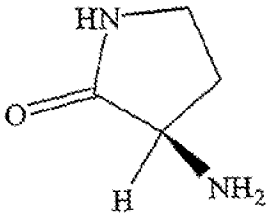


ISP1 or myriocin



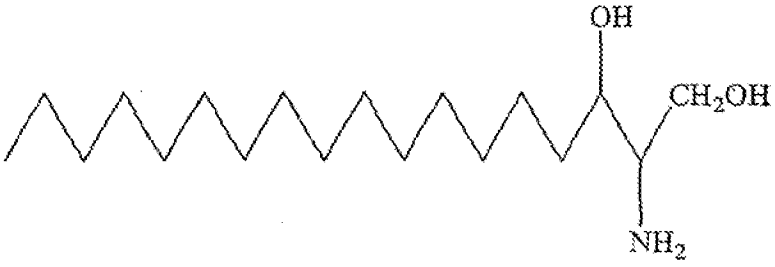
Lipoxamycin

Haloalanines



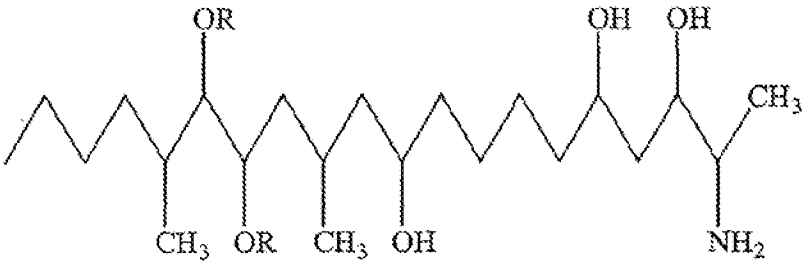
Cycloserine

Sphingosine kinase Inhibitor



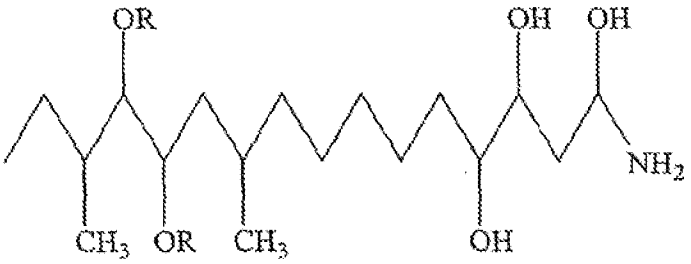
DL-threo-sphinganine

Ceramide Synthase Inhibitors



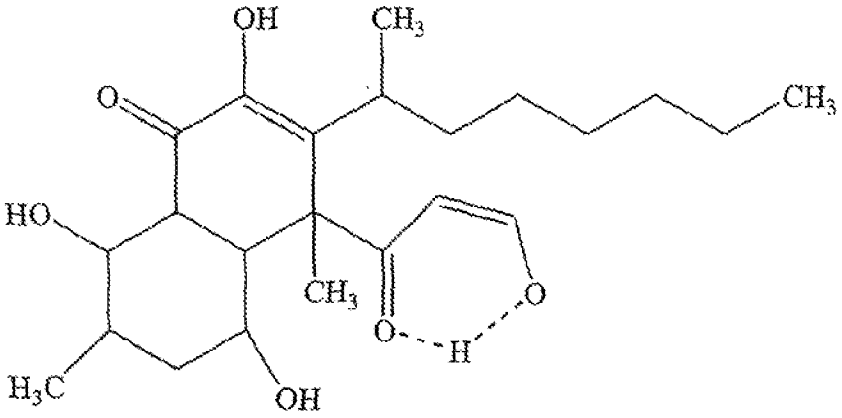
$R = COCH_2CH(COOH)CH_2COOH$

Fumosin B_1



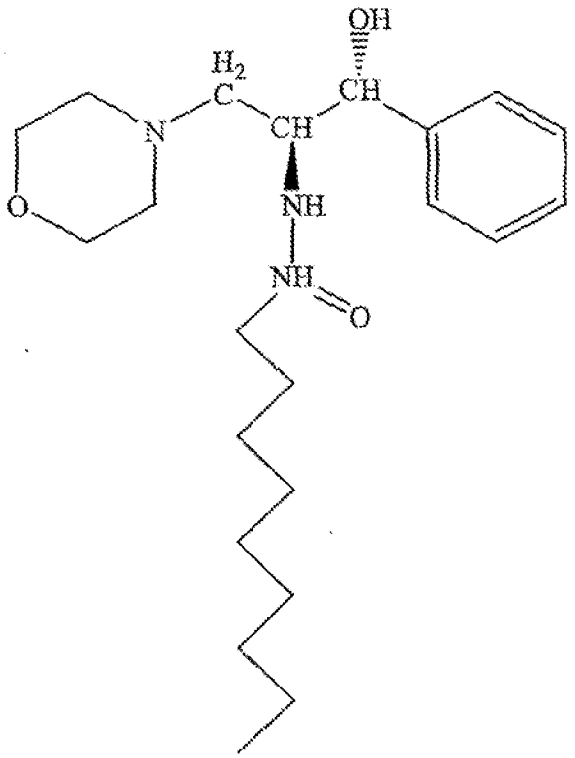
$R = COCH_2CH(COOH)CH_2COOH$

AAL-toxin



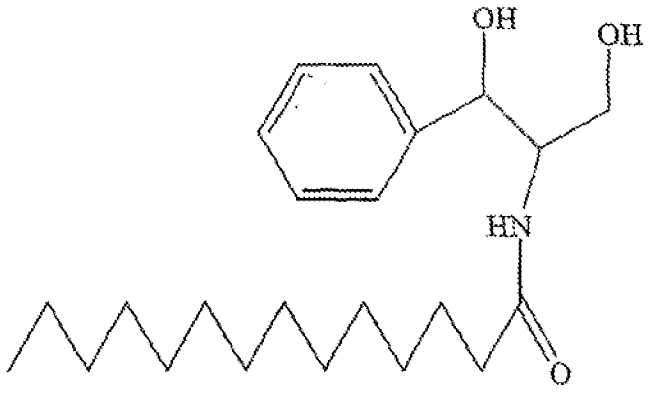
Australifungin

Cerebroside synthase Inhibitor



PDMP

Ceramidase Inhibitor



D-*erythro*-MAPP

Screening for a SM Biosynthesis Inhibitor

Isari sinclairii is a fungus traditionally used in Chinese medicine for "eternal youth". From *Isari sinclairii*, myriocin, a potent immunosuppresant and a specific serine palmitoyltransferase (SPT) (key enzyme for SM synthesis) inhibitor, was isolated. Myriocin has molecular structure similar to sphingosine. Using a myriocin-based affinity chromatography, only two proteins, LCB1 and LCB2, were purified from an IL-2-dependent mouse cytotoxic T cell line (CTLL-2) (Chen, Lane and Schreiber. 1999. Chem. Biol. 6:221-235). This result indicated that LCB1 and LCB2 are myriocin-binding proteins, and confirmed that they are responsible for the SPT activity.

Since *Myriococcum albonyces*, another fungus contains much more myriocin than *Isari sinclairii*, we chose *Myriococcum albonyces* to purify myriocin. After using the fungus fermentation, compound extraction and column chromatograph, we have successfully isolated myriocin with more than 95% purity.

In order to assess the inhibitory effect of myriocin on SPT, we utilized purified myriocin to perform sphingomyelin synthesis experiment, i.e. incorporation of ^{3}H-serine into sphingomyelin in tissue culture system. As shown in Figure 9A and 9B, both cellular and secretory ^{3}H-sphingomyelin were dramatically decreased in J774 macrophages after myriocin treatment. The IC_{50} was about 5μM. The same inhibitory effect was also observed in HepG2 cells, a liver cell line (Fig. 10A and 10B). Using homologous recombination techniques, we have successfully obtained heterozygous LCB2 gene knockout (LCB2+/-) mice. In order to know the consequence of one allele

SPT2 gene disruption, we have measured plasma SM levels in these animals and their wild type litter mates. As show in Table 2, plasma SM concentration in SPT2+/-mice were significantly lower than that of the control. There were no changes in plasma total phospholipid levels, thus SM/(SM+PC) ratio were significantly increased (Table 3). These results illustrate the utility of the SM assay in screening for drugs which inhibit the SM biosynthesis.

Sphingomyelin as a Target for
Known Cholesterol Lowering Drugs

The disclosed plasma sphingomyelin diagnostic assay can be used to assess the possibility that cholesterol lowering drugs, such as statins or fibrates, may lower plasma sphingomyelin levels. This could be part of the mechanism by which such agents offer protection against cardiovascular disease. These drugs lower the number of the particles in blood carrying cholesterol. Therefore, these drugs may also lower sphingomyelin levels of blood. For example, statins act by increasing the activity of LDL receptors in the liver; this may also lead to removal of sphingomyelin-rich lipoprotein particles from the circulation, as these particles are likely to be susceptible to clearance by the LDL receptor. However, the concept that cholesterol lowering drugs may also lower plasma sphingomyelin levels has never been previously tested.

Results and Discussion of Example 3

The foregoing shown that the disclosed method of measuring plasma sphingomyelin concentration in plasma and tissue is rapid and precise, with the ability to test a library of samples, and requiring only small amounts, e.g from 0.001 ml to 5.0ml of each sample.

Such an invention revolutionizes the possibilities for treatment of cardiovascular diseases. This innovative clinical diagnostic assay is a more efficient method than classical methods of SM measurement because in a shorter period of time, it can determine which agents reduce sphingomyelin levels in a subject.

The assay for sphingomyelin can be used to screen for inhibitors of sphingomyelin biosynthesis or for inducers of sphingomyelin clearance and thus is a useful tool for developing the optimum treatment for cardivascular disorders in a subject. This would be done by growing cells, for example, liver cells or liver cell lines in cell culture. Other cell types such as macrophages could also be used. These cells synthesize sphingomyelin and secrete the sphingomyelin into the culture medium. Thus, cells would be grown in multi-well plates and potential inhibitors would be added to the cells. Then the medium would be collected from the cells and assayed for its content of sphingomyelin. Also, the assay could be used in animal models that were injected with or fed potential sphingomyelin inhibitors. Plasma would be collected from the animals and assayed for sphingomyelin content.

Another possible use for the assay is as a good diagnostic test for early detection of coronary artery stenosis and atherosclerotic disorders. The assay may be utilized to periodically measure SM levels in a subject. An increase in SM levels in the subject serves as an early indication of possible development of coronary artery stenosis and atherosclerotic disorders.

Sphingomyelin as a marker for the clearance of atherogenic remnants of triglyceride-rich lipoprotein

We have observed that postprandial sphingomyelin levels increase 2-3 fold within 3-6 hours and return to basal levels after 10 hours (Figure 7). This pattern is similar to triglyceride. Since SM is not degraded in plasma, it tends to become enriched in atherogenic remnants of triglyceride-rich lipoproteins. Several lines of evidence suggest that remnants of triglyceride rich lipoproteins are particularly atherogenic, but the relevant fraction of plasma lipoproteins has been difficult to measure. In part plasma SM measurements acts as a marker of atherogenic remnant, which accumulate in the postprandial state. They also indicate lipoprotein susceptibility to arterial wall sphingomyelinase.

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What is claimed is:

1. A method for determining sphingomyelin concentration in a plasma or a tissue comprising the steps of:

   (a) measuring an absorption spectrum of the plasma or tissue; and

   (b) calculating the concentration of sphingomyelin from said measured absorption spectrum using calibration coefficients determined from a calibration set comprising absorbance spectra wherein the spectra of the reporter or molecule of said calibration set are varied by concentration,

thereby determining sphingomyelin concentration in the plasma or the tissue.

2. The method of claim 1, wherein said absorption spectrum is measured in a wavelength appropriate for the peak absorption of the reporter molecule in the visible wavelength region.

3. The method of claim 1, wherein said absorption spectrum is measured in the ultra-violet visible wavelength region.

4. The method of claim 1, wherein said absorption spectrum is measured utilizing a radioactive tracer as a detector substance.

5. A method for measuring an amount of sphingomyelin in a sample comprising the steps of:

(a) treating the sample containing sphingomyelin with a sphingomyelinase to produce phosphorylcholine and N-acylsphingosine;

(b) adding a phosphatase to the phosphorylcholine produced in step (a) to remove phosphate and generate a choline;

(c) reacting the choline of step (b) with suitable catalysts and suitable agents to form a chromogen; and

(d) measuring the optical density of the chromogen produced in step (c);

wherein the amount of sphingomyelin is determined by comparing the optical density obtained in step (d) with an optical density of a standard solution of sphingomyelin which generated a linear response over a concentration range.

6. The method of claim 5, wherein the sample in step (a) is plasma or tissue sample.

7. The method of claim 5, wherein the sphingomyelinase in step (a) is selected from a group consisting of mammalian, eukaryotic, and bacterial sphingomyelinase.

8. The method of claim 7, wherein the sphingomyelinase in step (a) is bacterial sphingomyelinase.

9. The method of claim 5, wherein the phosphatase in step (b) is selected from a group consisting of bacterial, eukaryotic, and alkaline phosphatase.

10. The method of claim 9, wherein the phosphatase in step (b) is alkaline phosphatase.

11. The method of claim 5, wherein the suitable catalysts in step (c) is choline oxidase or a peroxidase.

12. The method of claim 5 conducted in an oxygen containing atmosphere, wherein the suitable agents in step (c) is oxygen, 4-aminoantipyrine, and phenol.

13. The method of claim 5, wherein the chromogen produced in step (c) is red quinone pigment.

14. A method for measuring an amount of sphingomyelin in a sample comprising the steps of:

(a) treating the sample containing sphingomyelin with a sphingomyelinase to produce phosphorylcholine and N-acylsphingosine;

(b) adding a phosphatase to the phosphorylcholine produced in step (a) to remove phosphate and generate a choline;

(c) adding a first suitable catalyst and oxygen to the choline produced in step (b) to generate hydrogen peroxide;

(d) adding a second suitable catalyst, a 4-aminoantipyrine, and phenol to the hydrogen

peroxide produced in step (c) to generate a chromogen; and

(e) measuring the optical density of the chromogen produced in step (d);

wherein the amount of sphingomyelin of step (a) is determined by comparing the optical density obtained in step (e) with an optical density of a standard solution of sphingomyelin which generated a linear response over a concentration range.

15. The method of claim 14, wherein the sample in step (a) is plasma or tissue sample.

16. The method of claim 14, wherein the sphingomyelinase in step (a) is selected from a group comprising mammalian, eukaryotic, and bacterial sphingomyelinase.

17. The method of claim 14, wherein the phosphatase in step (b) is selected from a group comprising bacterial, eukaryotic, and alkaline phosphatase.

18. The method of claim 14, wherein the first suitable catalyst in step (c) is choline oxidase.

19. The method of claim 14, wherein the second suitable catalyst in step (d) is peroxidase.

20. The method of claim 14, wherein the chromogen of step (d) is red quinone pigment.

21. The method of claim 14, wherein the optical density is between 480 - 510nm.

22. The method of claim 21, wherein the optical density is between 490~505nm.

23. A method for measuring an amount of sphingomyelin in a sample comprising the steps of:

(a) treating the sample containing sphingomyelin with a bacterial sphingomyelinase to produce phosphorylcholine and N-acylsphingosine;

(b) adding an alkaline phosphatase to the phosphorylcholine produced in step (a) to remove phosphate and generate a choline;

(c) adding a choline oxidase and oxygen to the choline produced in step (b) to generate hydrogen peroxide;

(d) adding a peroxidase, a 4-aminoantipyrine, and phenol to the hydrogen peroxide produced in step (c) to generate a red quinone pigment; and

(e) measuring the optical density of the red quinone pigment produced in step (d);

wherein the amount of sphingomyelin of step (a) is determined by comparing the optical density obtained in step (e) with an optical density of a standard solution of sphingomyelin which generated a linear response over a concentration range.

24. A method for determining whether a compound inhibits sphingomyelin biosynthesis or induces sphingomyelin clearance comprising the steps of:

(a) culturing cells which produce and secrete sphingomyelin in culture;

(b) measuring the sphingomyelin level in the culture according to the method of claim 1;

(b) administering the compound to be tested to the culture;

(c) measuring sphingomyelin level in the culture at various intervals of time after step (b) using the method of claim 1; and

(d) comparing the sphingomyelin level obtained in step (c) with the sphingomyelin level obtained in step (a), wherein the compound administered in step (b) inhibits sphingomyelin biosynthesis or induces sphingomyelin clearance if the sphingomyelin level measured in step (c) is lower than the sphingomyelin level measured in step (a).

25. The method of claim 24, wherein the cells are liver cells.

26. A method of treating an atherosclerotic disorder in a subject which comprises administering to the subject a pharmaceutical composition comprising an effective amount of a compound that reduces plasma sphingomyelin concentration.

27. The compound of claim 26, wherein the compound inhibits sphingomyelin biosynthesis or induces sphingomyelin clearance.

28. The method of claim 26, wherein the atherosclerotic disorder is coronary heart

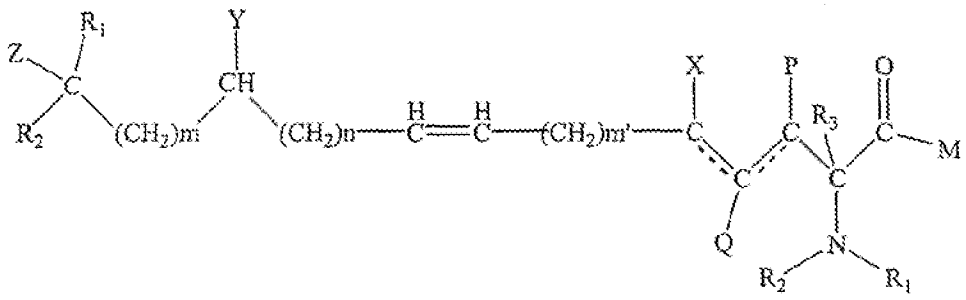
disease, hyperlipidemia, hypertriglyceridemia, familial hypercholesterolemia, atherosclerosis, or a renin/angiotensin control disorder.

29. The method of claim 26, wherein the compound is selected form the group consisting of ISP-1/myriocin, sphingofungin C, lipoxamycin, haloalanines, cycloserine, fumonisin B1, AAL-toxin, and australigungin.

30. The method of claims 26, wherein the compound is selected from a group consisting of a serine palmitoyltransferase inhibitor, a ceramide synthase inhibitor, a cerebroside synthase inhibitor, a shphingosine kinase inhibitor, and a ceramidase inhibtor.

31. The method of claim 30, wherein the compound inhibits serine palmitoyltransferase.

32. The method of claim 31, wherein the compound has the structure:



wherein each of R_1 and R_2 is the same or different and is hydrogen, or a C_1-C_4 substituted or unsubstituted hydrocarbon;

wherein R_3 is benzoyl or a halogen substituted benzoyl;

wherein Z is halogen, hydroxyl, amino, or C_1-C_4 substituted or unsubstituted hydrocarbon;

wherein each of m and m' is the same or different and is either 0 or 1, such that when m or m'is 0 then the respective (CH_2) group is absent;

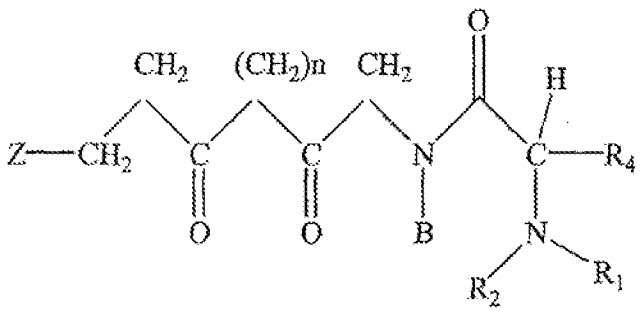
wherein n is an integer between 1 and 18;

wherein each of P, Q, X and Y is the same or different and is halogen, amino, or C_1-C_4 substituted or unsubstituted hydrocarbon;

wherein M is hydroxyl, amino, or C_1-C_4 substituted or unsubstituted hydrocarbon; and

wherein the dashed line represents a covalent bond that is either present or absent.

33. The method of claim 31, wherein the compound has the structure:



wherein each of R_1 and R_2 is the same or different and is hydrogen, or a C_1-C_4 substituted or unsubstituted hydrocarbon;

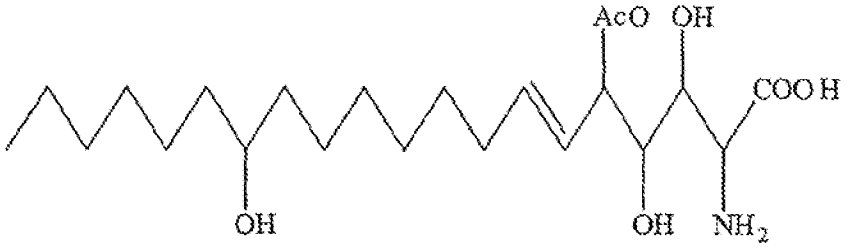
wherein R_4 is hydroxyl, amino, or C_1-C_4 substituted or unsubstituted hydrocarbon;

wherein Z is halogen, hydroxyl, amino, or C_1-C_4 substituted or unsubstituted hydrocarbon;

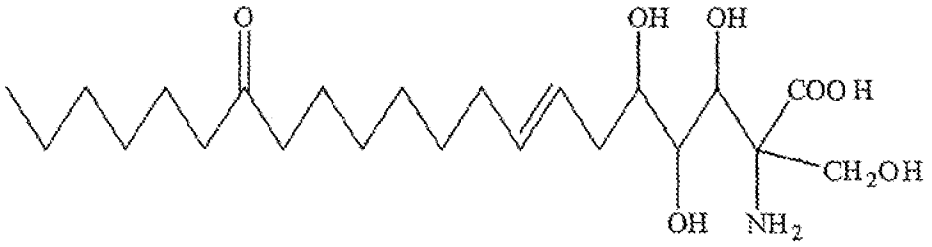
wherein n is an integer between 1 and 18; and

wherein B is halogen, amino, or C_1-C_4 substituted or unsubstituted hydrocarbon.

34. The compound of claim 32, having the structure:



35. The compound of claim 32, having the structure:



36. The compound of claim 33, having the structure:

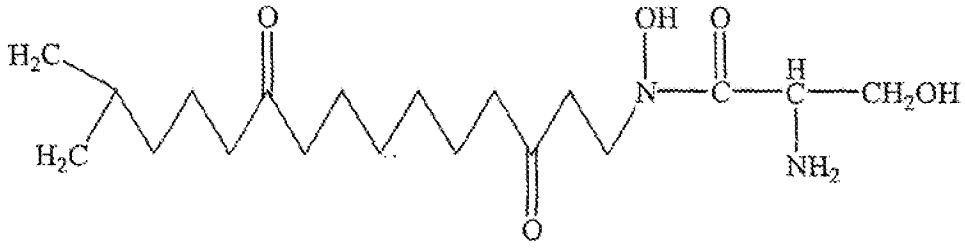


FIGURE 1

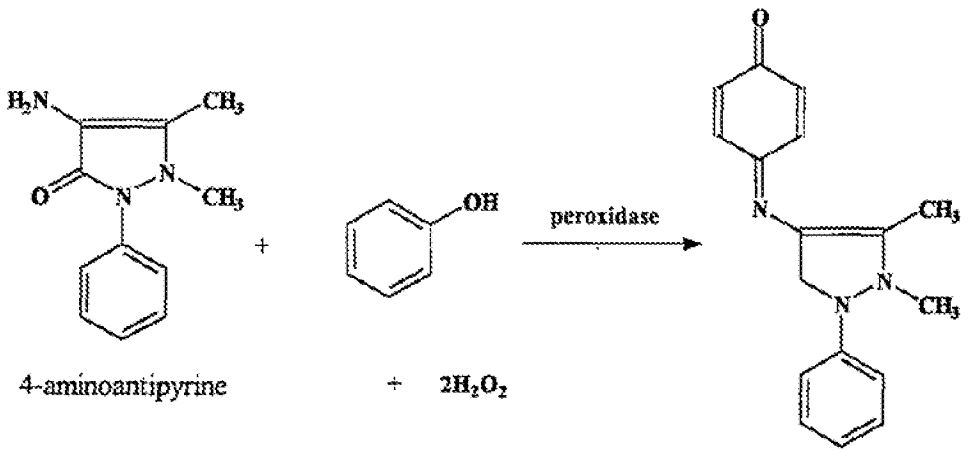
1.) Sphingomyelin $\xrightarrow{\text{sphingomyelinase}}$ phosphorylcholine + N-acylsphingosine

2.) phosphorylcholine $\xrightarrow{\text{alkaline phosphatase}}$ choline + phosphate

3.) $HOCH_2CH_2N(CH_3)_3^+CH^- + 2O_2 \xrightarrow{\text{choline oxidase}} 2H_2O_2 + {}^-OCCH_2N(CH_3)_3$

choline

4.)



4-aminoantipyrine + $2H_2O_2$

FIGURE 2

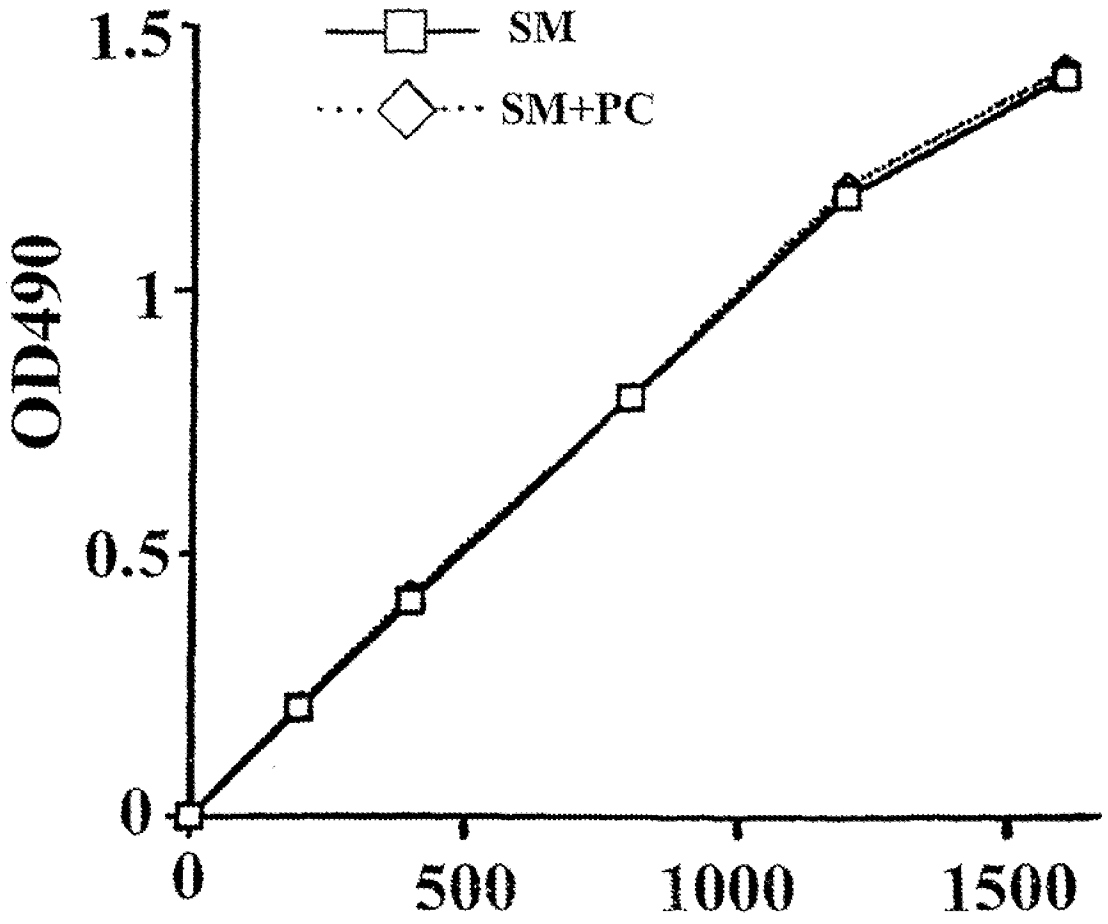


FIGURE 3

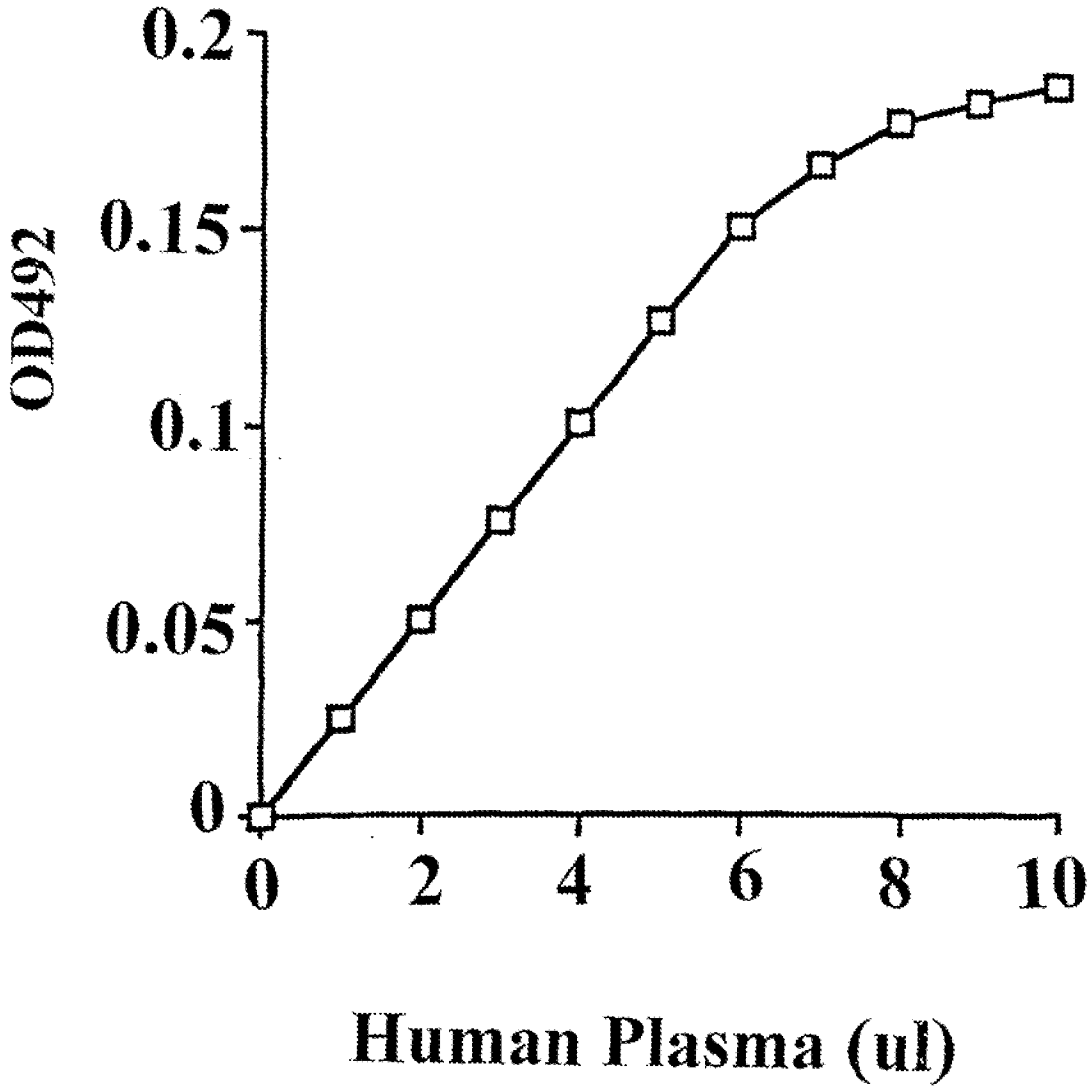
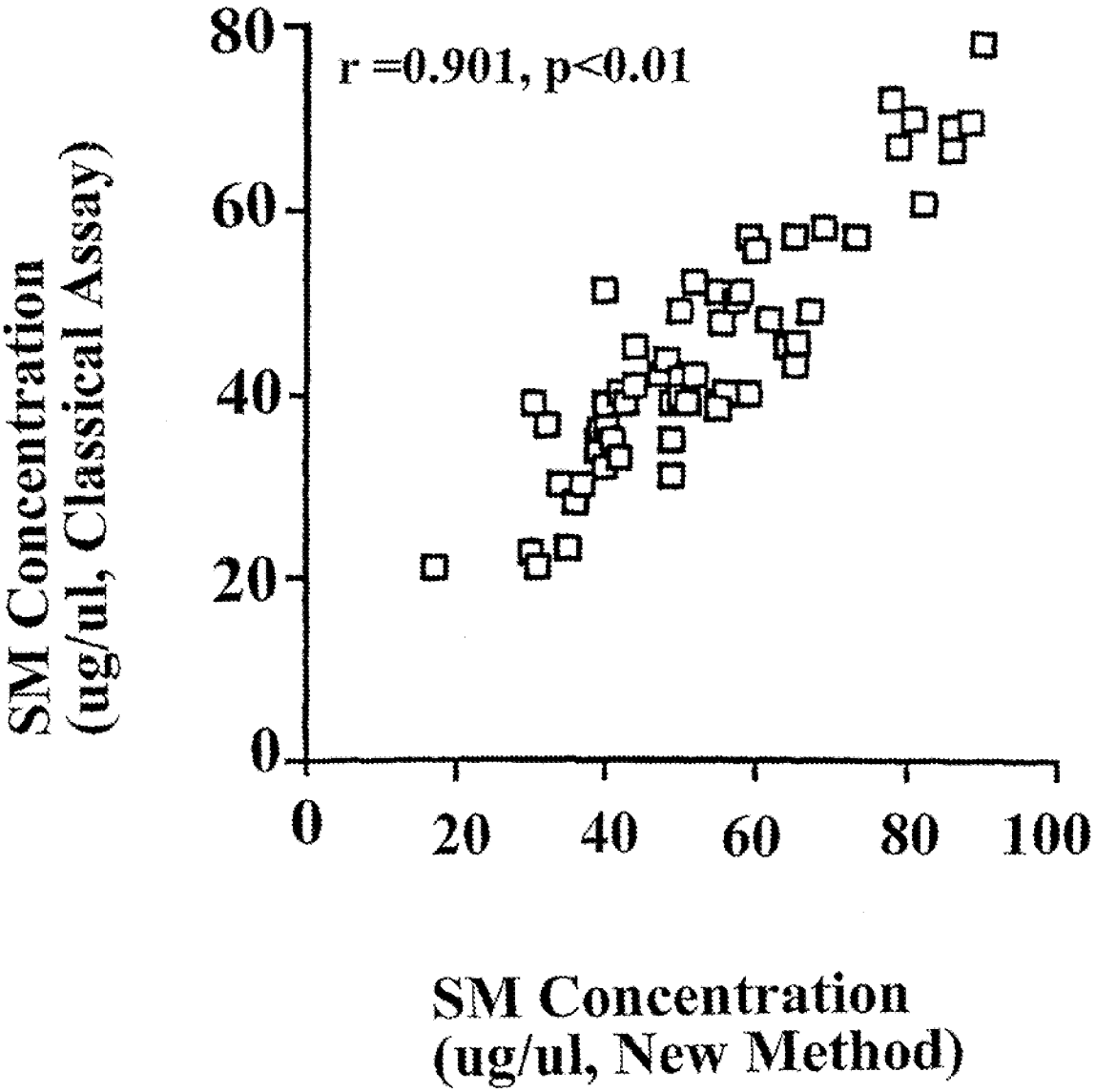


FIGURE 4


r =0.901, p<0.01
SM Concentration (ug/ul, Classical Assay)
SM Concentration (ug/ul, New Method)
0
20
40
60
80
100

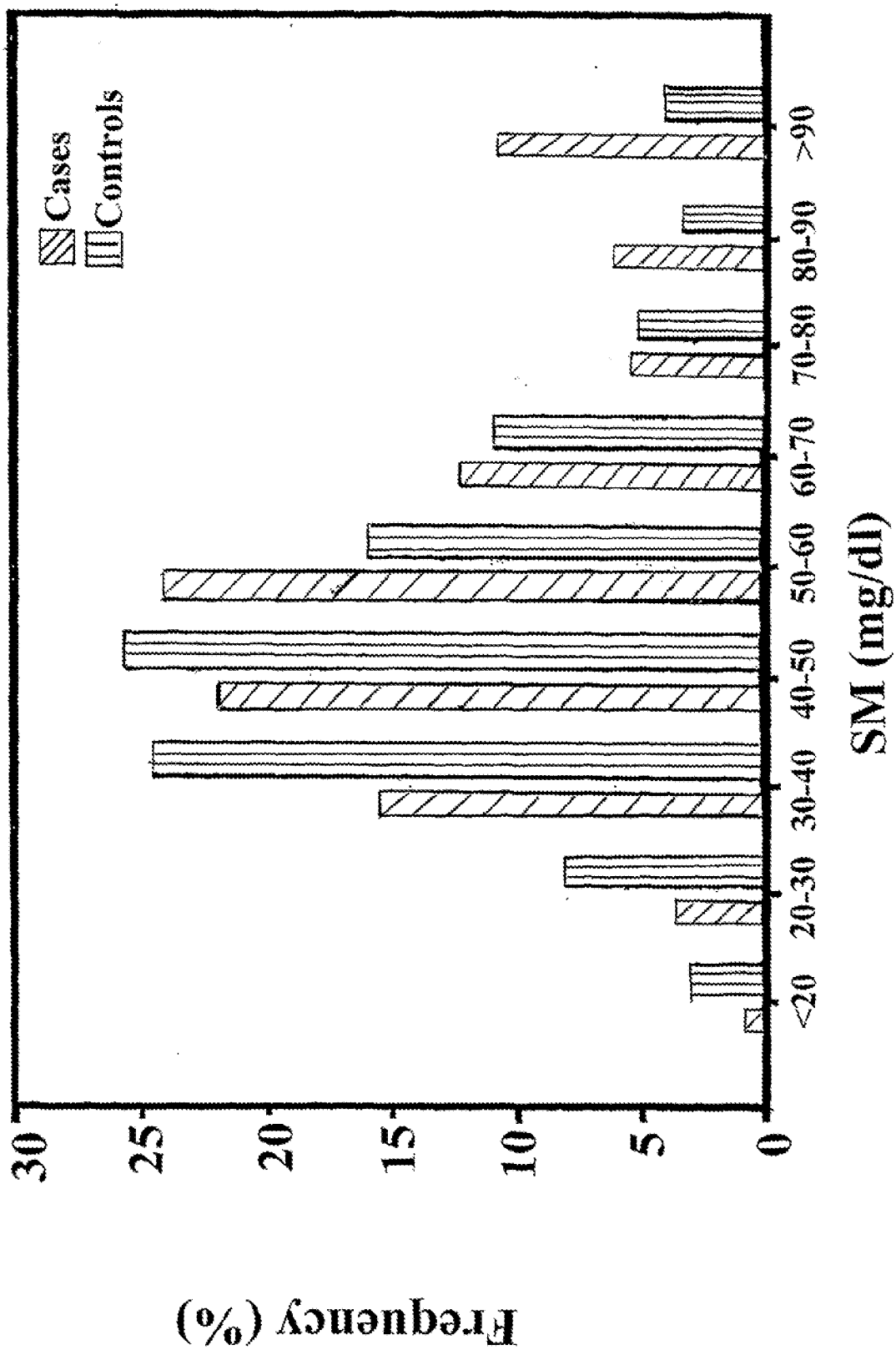

Cases
Controls
30
25
20
15
10
5
0
Frequency (%)
<20
20-30
30-40
40-50
50-60
60-70
70-80
80-90
>90
SM (mg/dl)

FIGURE 5

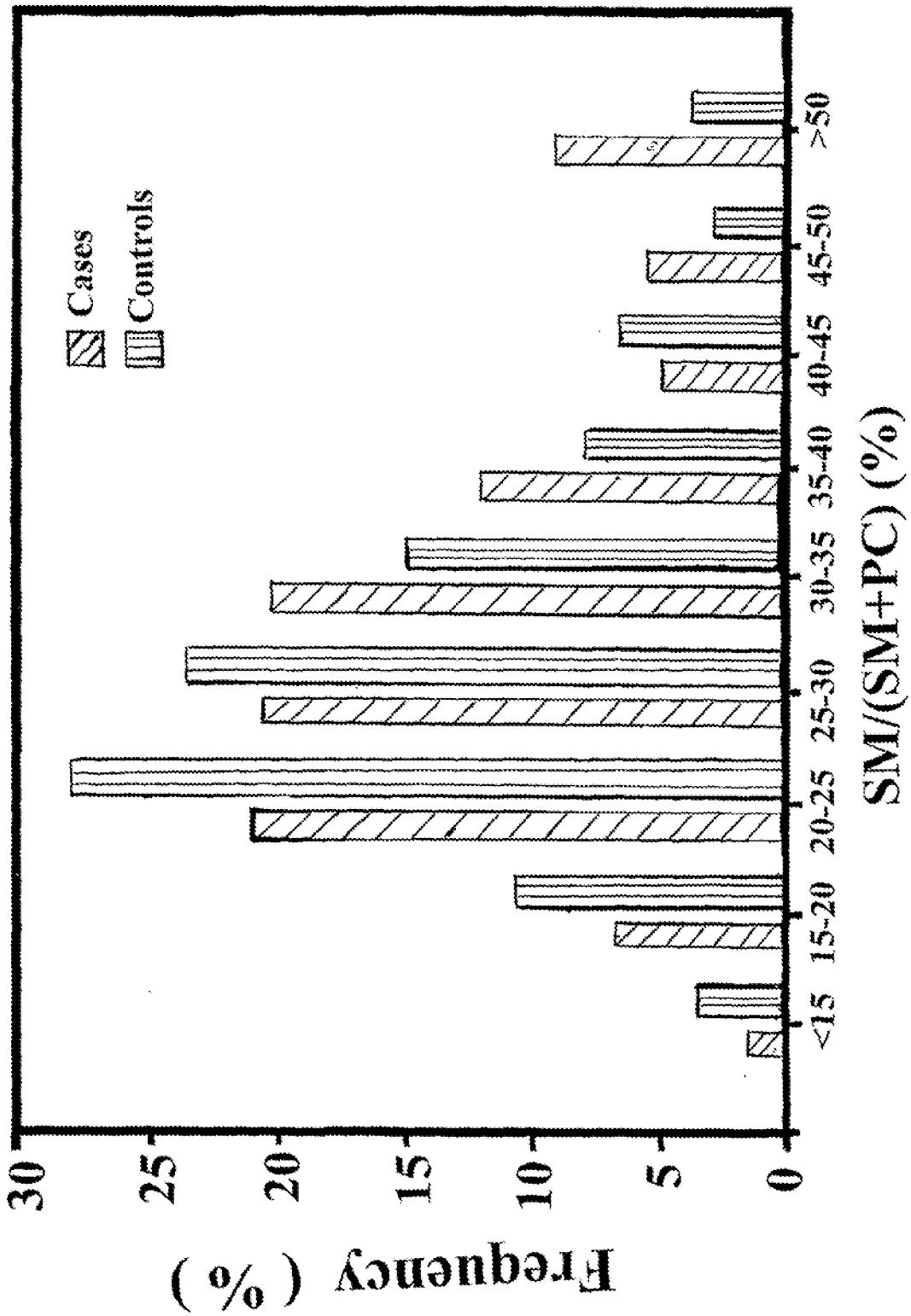

Cases
Controls
Frequency (%)
0
5
10
15
20
25
30
<15
15-20
20-25
25-30
30-35
35-40
40-45
45-50
>50
SM/(SM+PC) (%)

FIGURE 6

FIGURE 7

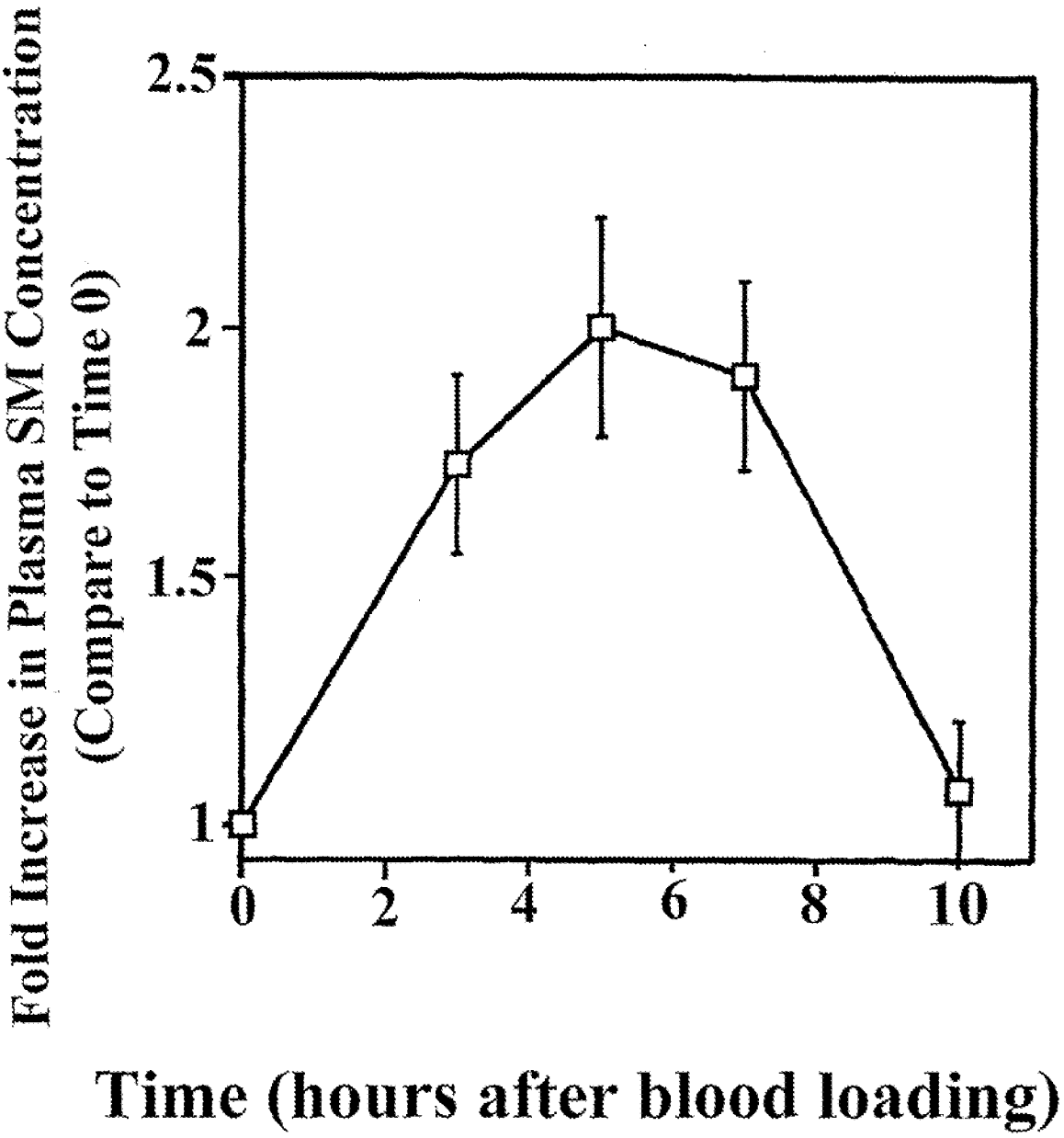
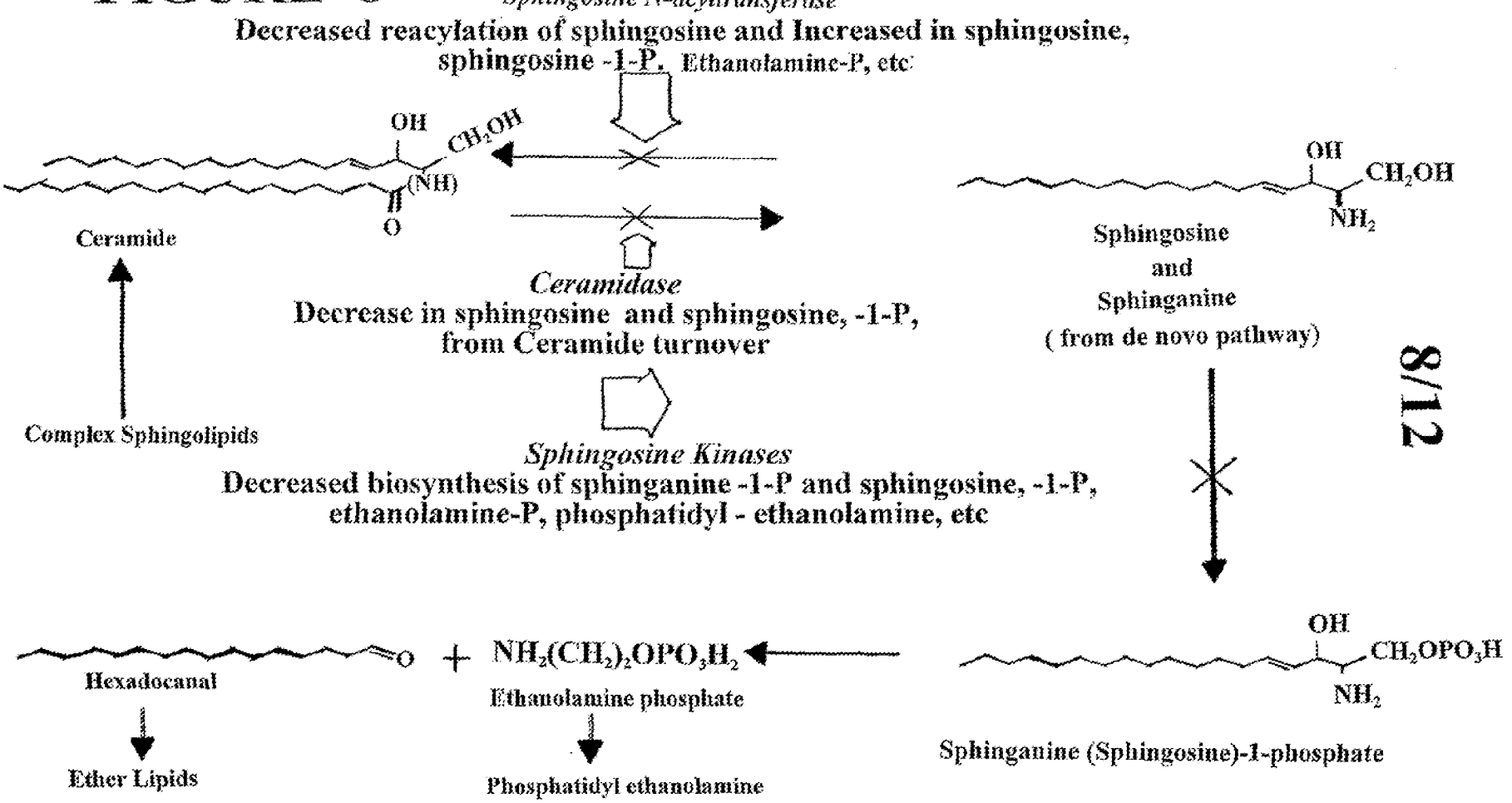

Fold Increase in Plasma SM Concentration
(Compare to Time 0)
2.5
2
1.5
1
0
2
4
6
8
10
Time (hours after blood loading)

FIGURE 8



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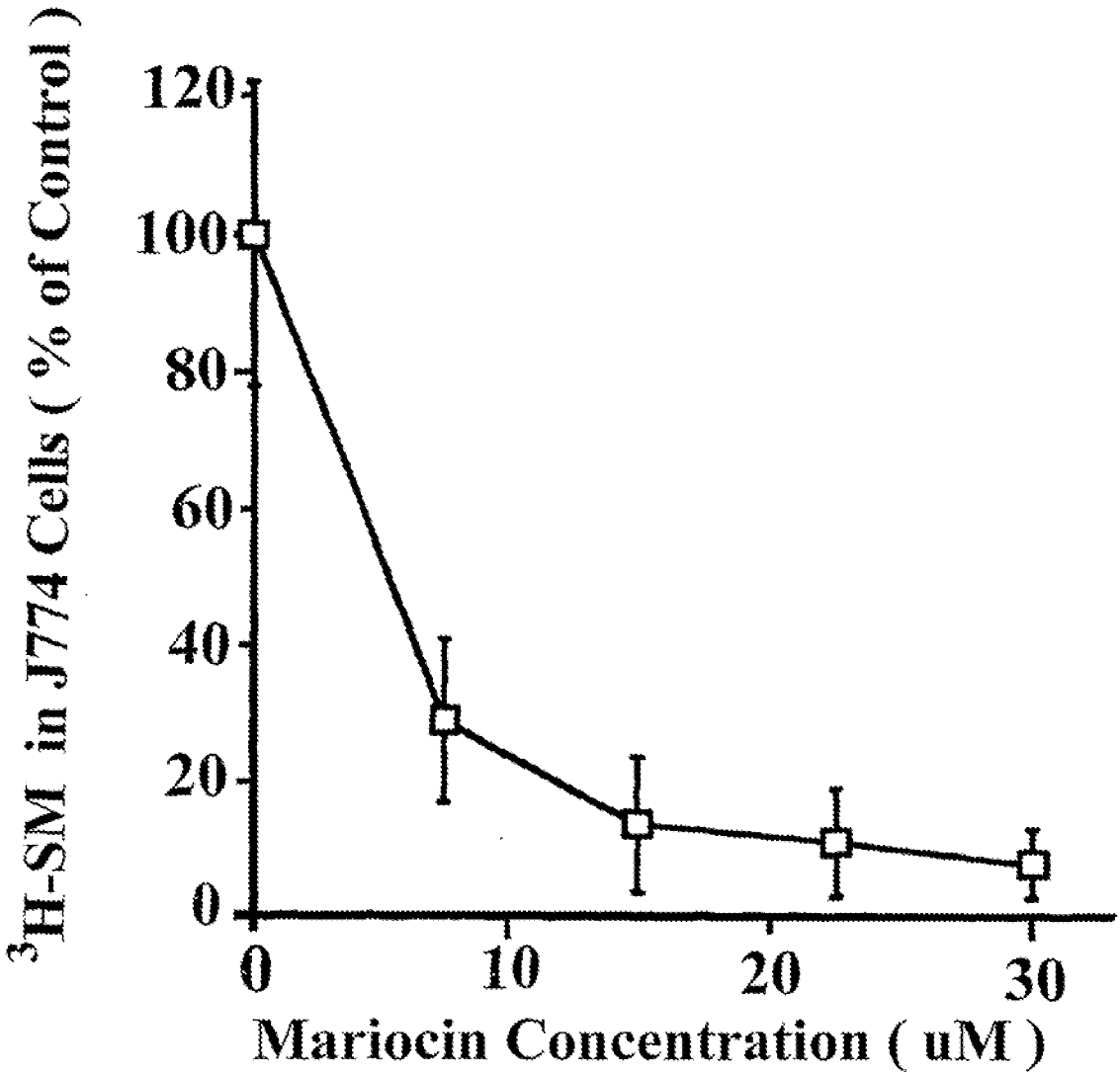
PCT/ES01/00170

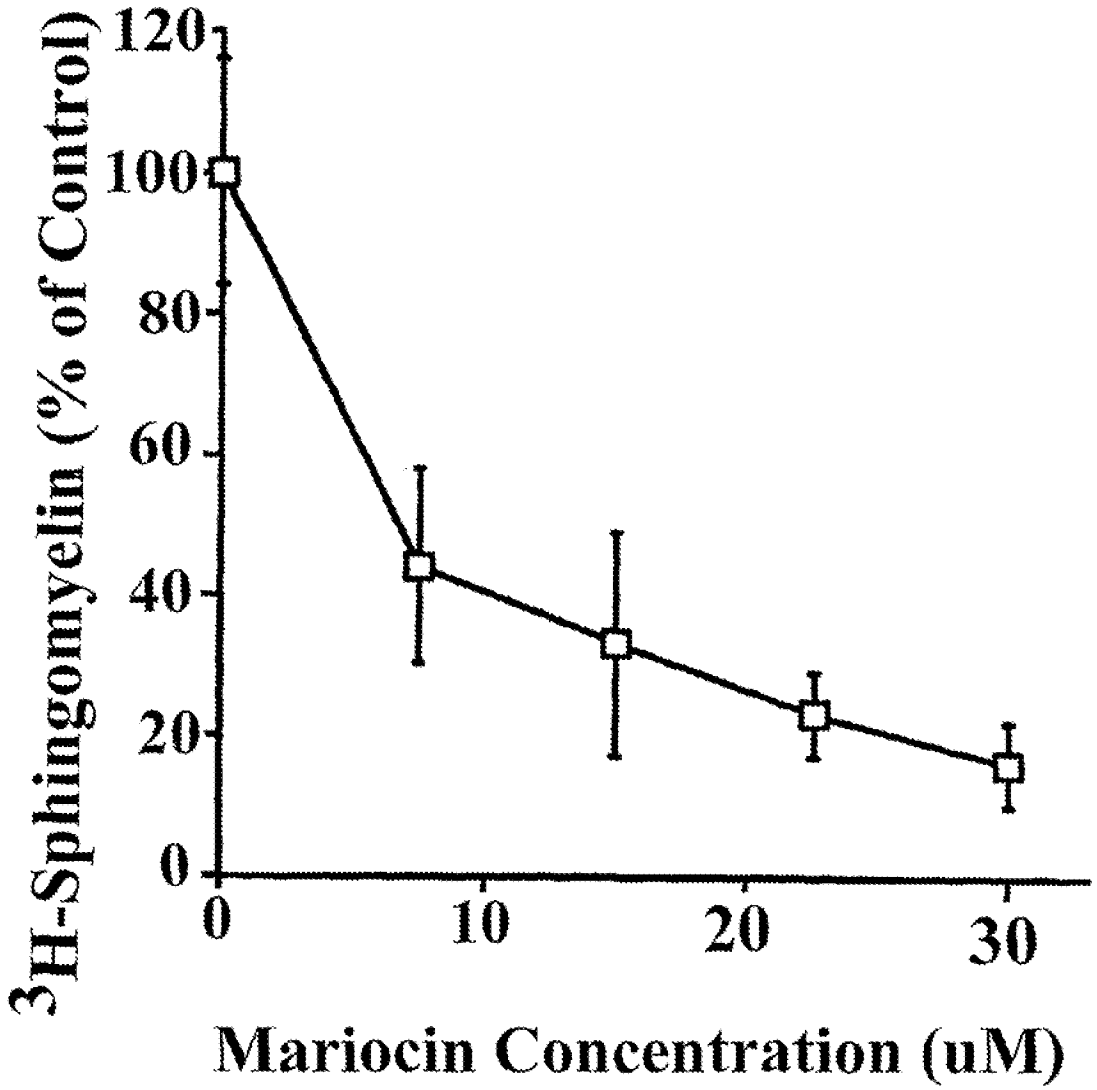
FIGURE 9A



Effect of Myriocin on Cellular Sphingomyelin Synthesis

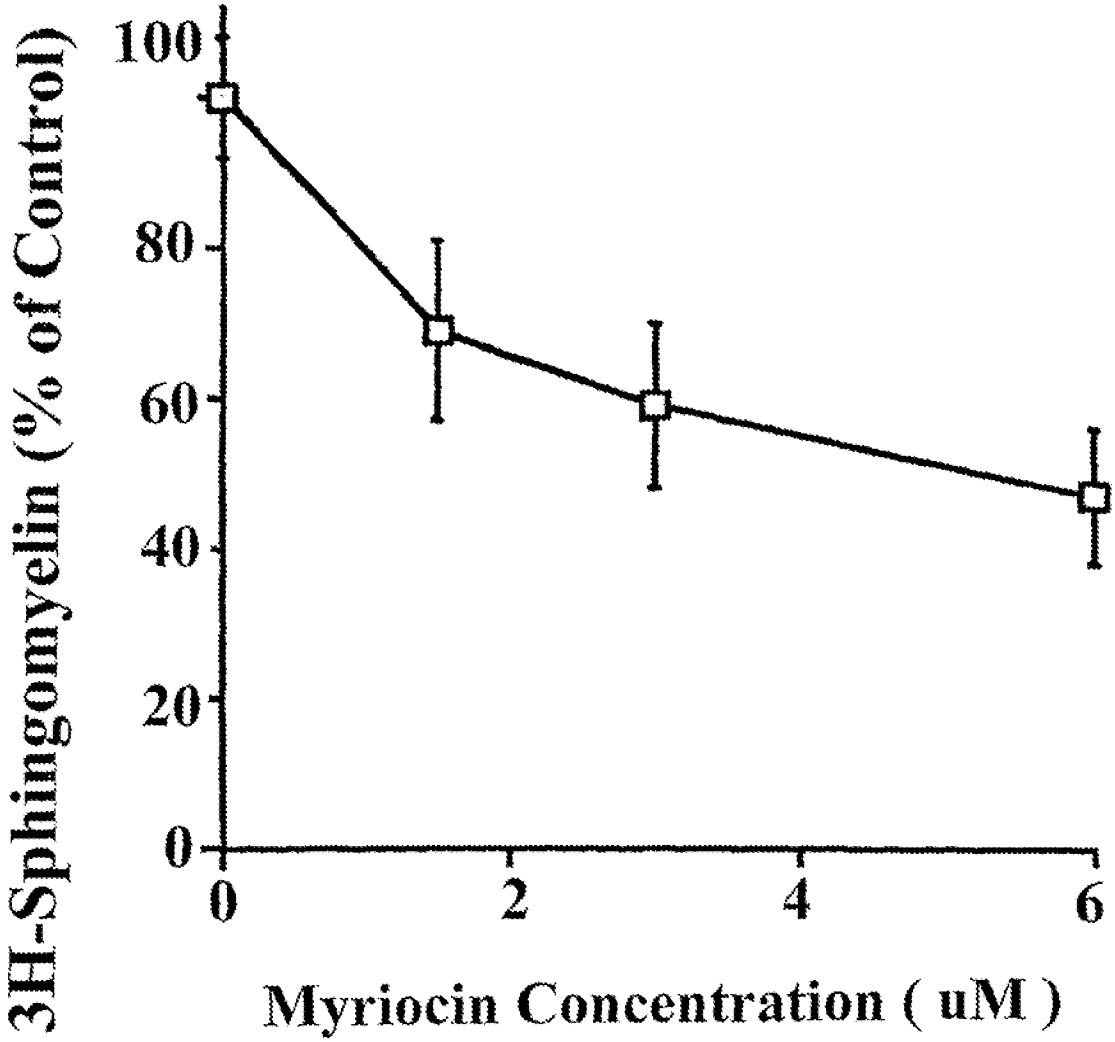
(^{3}H-Sphingomyelin in J774 Cells)

FIGURE 9B



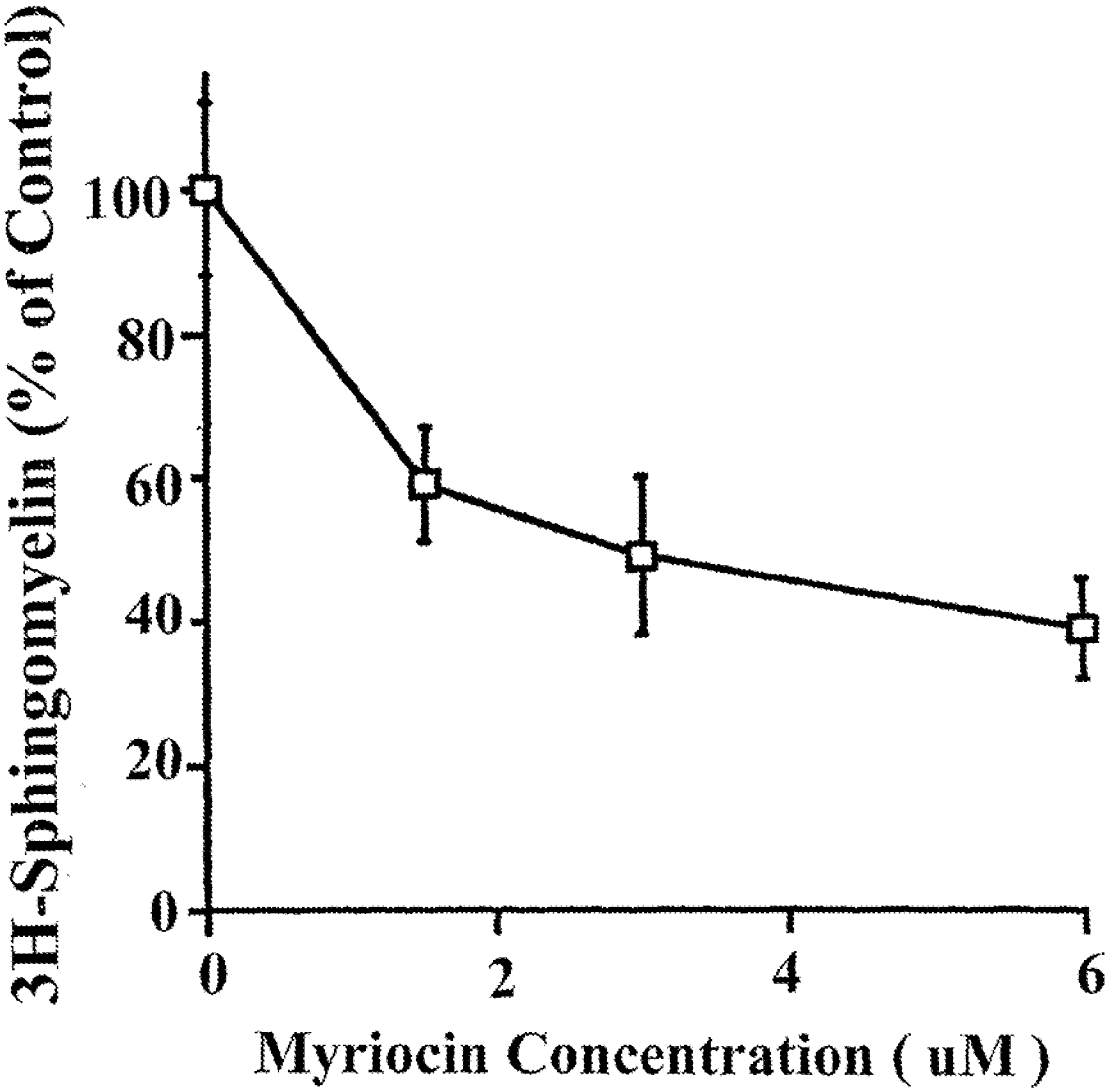
Effect of Myriocin on Sphingomyelin Secretion
(^{3}H-Sphingomyelin in the J774 Cell Medium)

FIGURE 10A



Effect of Myriocin on Cellular Sphingomyelin Synthesis
(^{3}H-Sphingomyelin in HepG2 Cell)

FIGURE 10B



Effect of Myriocin on Sphingomyelin Secretion
(^{3}H-Sphingomyelin in the Medium of HepG2 Cell)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/12706

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :A61K 49/00; C12Q 1/26, 1/44

US CL :424/9.1; 435/19, 25

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/9.1; 435/19, 25

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, CHEMICAL ABSTRACTS, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MCGOWAN M. W. A Procedure for the Determination of High Density Lipoprotein Choline Containing Phospholipids. J Clin Chem Clin Biochem 1982. Vol 20. No. 11. Pages 807-812, see especially page 808 column 1.	1-23
A	US 5,846,720 A (FOULKES et al.) 08 December 1998.	1-23
A	ENCINAR J. Enzymatic Determination of Phosphatydlcholine, Sphingomyelin and Phosphatidylglycerol in Lipid Dispersions, Blood Cell Membranes and Rat Pulmonary Surfactant. European J of Clinical Chemistry and Clinical Biochemistry. 1996. Vol. 34. No. 1, Pages 9-15.	1-23

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 27 JUNE 2001	Date of mailing of the international search report 29 AUG 2001
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